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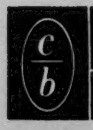

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The National Institutes of Health of the Public Health Service has supported in part the cost of publishing BIOCHEMISTRY through the purchase of copies for distribution to American medical libraries.

THE MICRODETERMINATION OF TOTAL NITROGEN BY THE NINHYDRIN METHOD

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Original article submitted January 7, 1960

In recent years, ninhydrin has found wide use both for qualitative and quantitative determination of various nitrogen containing compounds: amino acids [1], amino nitrogen of proteins and polypeptides [2], ammonia in microdiffusion [3], phosphatides [4], antibiotics [5], etc.

A study by Fels and Veatch [6] was recently published on the determination of protein nitrogen using ninhydrin. Independent of the latter authors we have made a study on the usefulness of the ninhydrin reaction for microdetermination of total nitrogen in mineralyzates.

EXPERIMENTAL

Principle of the method. The sample to be studied was mineralyzed by igniting with H_2SO_4 and the mineralyzate was carefully neutralized with NaOH. The amount of ammonia nitrogen was estimated by the intensity of the ninhydrin reaction colorimetrically.

Reagents and Technique of Determination

Ninhydrin reagent. a) Four g of ninhydrin, recrystallized and purified with activated charcoal [1], was dissolved in 100 ml of methyl cellosolve. If necessary, the latter was first purified from peroxides by heating on an air bath under reflux for 1.5 hr with $FeSO_4$ and Na_2CO_3 and then distilled. We took the fraction with b.p. 122-125°. The methyl cellosolve should not turn yellow when shaken with several drops of 5% KI; b) 0.32 g of $SnCl_2$ was dissolved in 100 ml of citrate buffer, pH 5.3 80.0 ml of 0.1 N sodium citrate and 20.0 ml of 0.1 N NaOH). Both solutions were combined in equal volumes. The concentration of ninhydrin in the reagent was 2%. The citrate buffer was prepared for storage and kept in a refrigerator; the ninhydrin solution was prepared on the day of the determination.

A standard solution of $(NH_4)_2SO_4$ contained 100 μg of nitrogen per ml (0.4719 g of recrystallized $(NH_4)_2SO_4$ in 1 liter of water distilled twice, free from NH_3). From this basic solution we prepared the working standards by dilution; they had a content of ammonia nitrogen from 1 to 10 μg for a calibration curve.

50% Aqueous ethanol. A sample of the material studied was ignited with 0.3-0.5 ml of concentrated H_2SO_4. At the end of the ignition 1-2 drops of perhydrol were added. The solution was neutralized to methyl red, and diluted to a nitrogen concentration of 1-10 μg per ml. We then took 1 ml in a tube with a mark at 10 ml, and added 1 ml of the ninhydrin reagent (final concentration of ninhydrin in the sample 1%). The tube was covered with aluminum foil and placed in a metal support in a strongly boiling water bath for 25 min, then transferred to a cold bath. The contents of the cooled tube were diluted with 50% aqueous ethanol to 10 ml, and the sample was measured in a photoelectric colorimeter with a yellow filter (λ_{max} 595 mμ) against the control reagent. In the absence of a yellow filter, the determination can be carried out with a green filter (λ_{max} 530 mμ), on which the sensitivity of the reaction is somewhat decreased, but the linear relation between concentration and optical density is maintained. The amount of nitrogen in the sample is determined by the standard curve and is calculated by the formula $N(\%) = a \cdot n \cdot 100/m \cdot 1000$, where a is the amount of nitrogen, μg; n the dilution; m the sample, mg.

Test of the Method

As is known, the colored complexes with amino acids are formed not with ninhydrin itself, but with the product of its reduction, hydrindantin. The latter is formed in the reaction of ninhydrin with amino acids, but a

quantitative yield of the colored product is found only with an excess of hydrindantin, which occurs only with introduction into the reagent of hydrindantin or with a reducing agent by which ninhydrin is converted into hydrindantin. As the reducing agent of ninhydrin and stabilizer of the colored complex, various authors have used $SnCl_2$ [1], ascorbic acid [7], KCN [8], and ethylenediamine tetraacetic acid [9]. The presence of a reducing agent is especially valuable for the determination of total nitrogen, since in the mineralyzate there is no natural antioxidant (amino acid) and the color reaction without a reducing agent practically does not develop [9].

As the basis for the ninhydrin reagent we took the reagent of Moore and Stein where, as the reducing agent for ninhydrin, such an available compound as $SnCl_2$ was used. In using the reagent of Moore and Stein [1, 3] the final concentration of $SnCl_2$ and ninhydrin in the reaction mixture remains the same as in the reagent itself [3], or is changed slightly to 1.8% ninhydrin [1].

Considering the twofold dilution of the ninhydrin reagent in our method (adding 1 ml of ninhydrin reagent to 1 ml of solution) we can expect that this will require an increased concentration of ninhydrin and $SnCl_2$, also twofold. However, our experiments showed that increase in ninhydrin concentration in the reagent had a negative effect on the sensitivity of the ninhydrin reagent (expressed in units of optical density), which was shown especially clearly in the absence of $SnCl_2$ in the reagent.

$(NH_4)_2SO_4$, μg/ml	$SnCl_2$, %	Ninhydrin, % 2,0	1,0	0,5
10,0	0,08	0,690	0,712	0,630
»	—	0,218	0,299	0,021
5,0	0,08	0,353	0,378	0,318
»	—	0,052	0,123	0,024
1,0	0,08	0,073	0,080	0,076

One percent concentration of ninhydrin in the reaction mixture was optimal. Thus, on raising the ninhydrin concentration above the established level we found a decreased intensity of the ninhydrin reaction. It should be noted that this decrease was statistically valid even with such a strict criterion of significance as the average error of the difference between the two averages. A study of the effect of $SnCl_2$ concentration in the ninhydrin reagent on the sensitivity of the ninhydrin reaction with $(NH_4)_2SO_4$ shows the following:

Concentration of $SnCl_2$ in Reagent, %	Optical density
0,32	0,68
0,16	0,73
0,08	0,65
0,04	0,54
0,02	0,27

Thus, the optimum concentration of $SnCl_2$ corresponds to the expected value, that is, twice as high as in the reagent of Moore and Stein.

In a study of the effect of pH of the citrate buffer on the sensitivity of the ninhydrin reaction we obtained the following results:

pH of buffer	Optical density
5,0	0,25
5,1	0,26
5,2	0,29
5,3	0,35
5,4	0,31
5,5	0,22

Thus, the optimum pH of the citrate buffer at our optimum concentrations of ninhydrin and $SnCl_2$ was somewhat shifted to the alkaline side: 5.3 instead of 5.0.

Since in the neutralization of the mineralyzate, Na_2SO_4 is mixed with $(NH_4)_2SO_4$, we tested the effect of the former salt on the sensitivity of the ninhydrin reaction. We studied two concentrations of Na_2SO_4: one corresponding to the concentration in the neutralized mineralyzate, the other twice the first:

Expt. No.	N_2, g/ml	Na_2SO_4, mg%	Optical density	$\sigma\sqrt{\frac{n}{n-1}}$	$\sqrt{\frac{\sigma_1^2}{n_1}+\frac{\sigma_2^2}{n_2}}$	Coefficient variation
1	5,0	180,0	0,376	0,0011	0,0017	0,3
2	5,0	360,0	0,390	0,0110	0,0054	2,9
Control	5,0	—	0,375	0,0036	—	0,9

As these results show, Na_2SO_4 has no effect on the ninhydrin reaction with $(NH_4)_2SO_4$. Some increase in optical density in experiment 2 lay within the limits of three times the average error of the difference between the two averages.

The maximum color in the reaction of $(NH_4)_2SO_4$ with the ninhydrin reagent with intensive boiling of the water bath developed in 25 min (Fig. 1); boiling the sample more than 40 min resulted in marked decrease in color. Since the maximum in color shows a sharp peak, it is necessary to control very strictly the conditions of heating on the water bath.

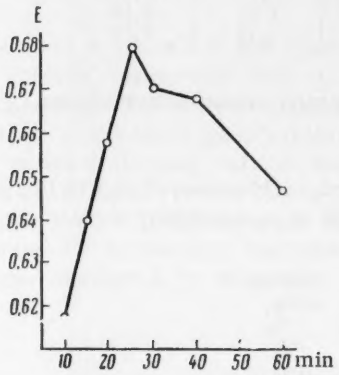
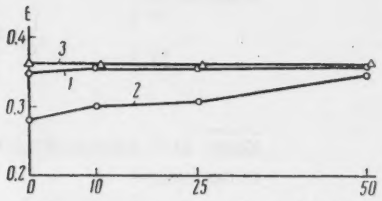
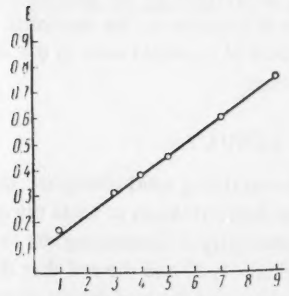


Fig. 1. Effect of length of heating on a boiling water bath on intensity of ninhydrin reaction.



Concentration of aqueous ethanol solution, %

Fig. 2. Effect of concentration of aqueous ethanol solution on intensity of ninhydrin reaction. 1) sample diluted immediately after reaction on the water bath and measured colorimetrically at once; 2) sample diluted immediately after reaction on the water bath, but measured colorimetrically after 1.5 hr standing of the solution at room temperature; 3) sample diluted 1.5 hr after reaction on the water bath and measured colorimetrically at once.



μg, N_2

Fig. 3. Calibration curve of standard solution of ammonium sulfate.

After the reaction on the water bath had been carried out, the solution, diluted with 50% aqueous ethanol, remained clear for an indefinite period, and during 6.5 hr keeping in the refrigerator the intensity of its color did not change; after keeping in the refrigerator for a day there was a decrease of only about 5% in optical density. When the solution, diluted with alcohol, was kept at room temperature, the color gradually weakened, and the less the concentration of alcohol used for the dilution, the more marked was the weakening in intensity of color (Fig. 2). However, if after the reaction had been run on the water bath, the solution was kept at room temperature for a long time without dilution and was diluted only immediately before the colorimetric measurement, the optical density remained at the previous level, independent of whether the solution was diluted with alcohol of different concentrations or with distilled water. Thus, if the solution was diluted directly before colorimetric measurement, 50% alcohol could be successfully replaced by water. The control for the reagent in a long series of experiments should best be diluted with 50% ethanol considering the long keeping of the diluted control in the cuvette.

Determination of Total Nitrogen by Kjeldahl and Ninhydrin Methods in Biological Materials.

Material studied	Portions of mineralyzate taken in the sample		N, %	
	Ninhydrin method	Kjeldahl method	Ninhydrin method	Kjeldahl method
Bacterial mass:				
P. pestis 773	1/400	1/10	12,65	12,55
	1/400	1/10	12,69	12,55
P. pestis 17	1/400	1/10	12,37	12,87
	1/400	1/10	12,15	12,12
P. pseudotuberculosis	1/500	1/10	10,32	10,12
	1/500	1/10	10,33	10,08
	1/500	1/10	9,94	10,23
Br. abortus	1/500	1/10	12,50	12,30
	1/500	1/10	12,41	12,58
Sheep serum:	1/250	1/10	0,80	0,86
№ 1	1/250	1/10	0,90	0,89
	1/250	1/10	0,91	0,86
№ 2	1/250	1/10	1,13	1,15
	1/250	1/10	1,13	1,11

Note: Each number is an average of results of several parallel determinations of one sample.

The limits of the measurement using a cuvette with a working edge of 10 mm were from 1 to 10 μg of nitrogen (Fig. 3). Study of the effect of concentration of nitrogen in the sample on reproducibility of results showed the following:

N, μg/ml	Optical density	$\sigma\sqrt{\frac{n}{n-1}}$	Relative error, %
2,0	0,152	0,0037	2,43
5,0	0,376	0,0027	0,71
10,0	0,657	0,0076	1,15

In order to test the accuracy of the method, we determined, in the same mineralyzate, the nitrogen content by the ninhydrin method and according to Kjeldahl (Table). For determination of nitrogen by Kjeldahl we took 1-2 ml of solution of the first dilution. The amount of distilled ammonia was determined by the amount bound by 0.01 N H_2SO_4 as titrated with 0.01 N NaOH. As the table shows, determination of nitrogen by the ninhydrin method gives good agreement with the results from the Kjeldahl method, but the amount of material used in the sample for the ninhydrin method is 25-50 times less than that used in the Kjeldahl method.

DISCUSSION OF THE RESULTS

The present work is a development of the ninhydrin method for determining total nitrogen. In the course of testing the usefulness of the ninhydrin reagent of Moore and Stein [1] for determination of total nitrogen, we made some of the conditions for applying it more accurate. We showed the possibility of decreasing the concentration of ninhydrin in the reaction mixture twofold compared to that of other authors [1, 3] and showed that this not only did not decrease the sensitivity of the reaction, but on the contrary increased it, and the variability of the determination was decreased. The optimum concentration of the reducing agent ($SnCl_2$) in the reagent was twice that in the reagent of Moore and Stein, and the optimum pH of the reaction was somewhat shifted to the alkaline side (5.3 instead of 5.0).

Fels and Veatch [6] used a citrate buffer of high concentration (0.5 M) to lessen the effect of Na_2SO_4 on the ninhydrin reaction with ammonium sulfate, but in this case they found a decrease in optical density of 8%. Under our optimum conditions (0.1 M citrate buffer, pH 5.3) Na_2SO_4 did not affect the intensity of the ninhydrin reaction.

The maximum intensity of the reaction with heating on a water bath was reached after 25 min and the color which developed remained constant for several hours, which permitted simultaneous running of a large series of

reactions. The solutions here not only retained the color, but remained clear. Dilution of the samples before colorimetric measurement could be carried out with distilled water instead of 50% ethanol.

The method has a high sensitivity; it gives the possibility of determining from 1 to 10 μg of ammonia nitrogen in the sample (using a cuvette with working edge 10 mm). The most accurate determination is found in the middle portion of the curve. The coefficient of variation is 0.9%. The method can be used for determination both of total and residual nitrogen, with the difference that in the second case the protein is first removed by one of the usual methods.

SUMMARY

We have suggested a modification of the colorimetric micromethod for determination of total nitrogen based on the ninhydrin reaction. The method is characterized by simplicity and rapidity of performance, high sensitivity, and reproducibility. The relative error of the colorimetric determination on an FEK-M photoelectric colorimeter is 0.7-2.3%.

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A STUDY OF THE RESYNTHESIS OF DEOXYRIBONUCLEIC ACID IN ISOLATED CELL NUCLEI

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Original article submitted July 1, 1960

In the studies of Allfrey and co-workers [1] it was established that nuclei isolated from half thymus could take up labeled amino acids; evidence was given in favor of the fact that the uptake of the labeled amino acids reflected the synthesis of nuclear proteins and not adsorption or nonspecific exchange reactions of amino acids. After partial depolymerization of DNA in the nuclei with DNA-ase, the uptake by amino acids actually fell; later incubation of such nuclei with homologous DNA restored their ability to synthesize protein almost to the starting level. Later the same authors [2], and, independently, one of us [3] showed that the effect was restored not only with homologous, but also with foreign DNA, and in the experiments of Allfrey and co-workers, also with RNA. In this connection the opinion was expressed of the possibility of replacing thus in the nuclei the individual depolymerized DNA by foreign DNA or by RNA and the possibility of introducing in this way new genetic information and thus inducing the synthesis of proteins foreign to the nuclei [2, 3].

However, this suggestion was quickly abandoned, since new facts raised doubts as to the value for species specificity of the portion of DNA destroyed by DNA-ase. It was shown that the synthesis of protein in such nuclei could be restored not only by addition of DNA or RNA, but also by addition to the incubation medium of nonnucleotide polyanions such as chondroitin sulfate, polyethylene sulfate, or heparin [4]. These results led the authors to the conclusion that the depolymerized DNA in the nuclei could be replaced by foreign DNA or RNA and by other polymers which had negative charges like nuclei acids, and that the latter fact plays an important role in their functional activity (particularly in the restoration of ATP synthesis [5], a process which is known to be very important for protein synthesis). However, in the work discussed there was no direct study of the possibility of penetration into the nuclei in unchanged form of nucleic acids or other polyanions added from without. The conclusion of the penetration into the nuclei of these polymers was made chiefly on the basis of indirect data on their stimulating effect on protein resynthesis. The question of the actual possibility of penetration of foreign DNA, RNA, and nonnucleotide polyanions into the nuclei which have lost part of their own DNA, and their fulfilling the function of the latter, remains unsettled; the present work is devoted to this question.

METHODS

Nuclei were isolated from calf thyroid glands in isotonic sucrose media by the method of Stern and Mirsky [6], somewhat modified by us [3]. The isolated nuclei were suspended in 0.25 M sucrose with 0.003 M $CaCl_2$ (250 mg of nuclei per ml) and to 1 ml of the suspension was added 0.5 ml of 0.1 M sodium phosphate buffer in 0.25 M sucrose (ph 7.3) and 0.4 ml of 0.1 M glucose containing 8.16 mg NaCl and 4.19 mg $MgCl_2$ per ml. For depolymerization of nuclear DNA we added DNA-ase (0.4 mg per 10 mg of nuclei) to the mixture and incubated at 37° for 30 min; the nuclei were separated by centrifuging (10 min, 600 g) and again suspended in the incubation medium to which was added DNA (homologous or foreign), or RNA, or chondroitin sulfate, 2 mg/100 mg nuclei. To inhibit the action of DNA-ase in some of the experiments we added to the medium sodium citrate (20 μ mole). After 60 min, of second incubation with the polymer, the nuclei were again separated by centrifuging, washed with 0.25 M sucrose with 0.003 M $CaCl_2$ and studied biochemically and cytochemically; samples for study were taken at various stages of the process. We determined the content of acid soluble nucleotides in the supernatant liquid after the first and second incubations of the nuclei, and also in the nuclei themselves. The nuclear nucleic acids were separated by the modified method of Schmidt-Tannhauser-Schneider (instead of TCA we used perchloric acid) [7]; DNA was determined according to Barton [8]; RNA according Dische and Borenfreund [9]; chondroitin sulfate by the content of glucosamine [10].

TABLE 1. Effect of Added DNA, RNA, and Chondroitin Sulfate on Restoration of DNA in DNA-ase Nuclei

Experimental conditions	DNA content, mg/g nuclei		
	Expt. 1	Expt. 2	Expt. 5
Intact nuclei	30.8	45.2	30.0
Nuclei incubated with DNA-ase (30 min, 37°)	12.0	14.6	15.8
Incubated DNA-ase nuclei with one of the additives in the medium of substrate (60 min, 37°):			
Calf thyroid DNA	15.6	21.0	19.5
Rabbit DNA	15.5	–	–
Rat liver RNA	–	18.0	–
Chondroitin sulfate	–	–	17.6

Acid soluble nucleotides were determined by the color reaction for pentoses [8, 9] and also by the extinction in ultraviolet light (260 mμ) on an SF-4 spectrophotometer; for the determination of ribonucleotides by ribose they were first freed from the sucrose incubation medium by adsorption on carbolene with later elution by 40% alcohol containing 1% ammonia [11]. The preparation of high polymer RNA was obtained from rat livers [12], the DNA preparation from calf thyroid and rabbit liver and kidney [13], chondroitin sulfate from swine tracheas [14]. The admixture of RNA in the DNA preparation, after purification by alkaline hydrolysis [15], did not exceed 1%, and the admixture of DNA in the RNA preparation, 0.1%. The enzymatic hydrolyzates of DNA and RNA were obtained by incubation of DNA with DNA-ase (10 mg per 100 mg DNA) and RNA with RNA-ase (10 mg per 200 mg RNA) for 12 hr at 37° and later dialysis; the dialysates were brought to the desired concentrations.

RESULTS AND DISCUSSION

Under the action of DNA-ase the nuclei lose 50-80% of their DNA; on incubation of such nuclei with homologous or foreign DNA the content of DNA in the nuclei is restored, though it is true, not more than 8-25%.

In Table 1 we give typical experiments from which it is clear that addition of foreign (rabbit) DNA restores a quantity of DNA in the nuclei of calf thyroid which has undergone the action of DNA-ase ("DNA-ase" nuclei) to almost the same extent as in the addition of homologous DNA.

In recently published investigations [16, 17] we showed that the introduction of RNA in the incubation medium with DNA-ase nuclei also caused increased content of DNA in them. One of such experiments is given in Table 1. It was shown that the addition to the DNA-ase nuclei of such nonnucleotide polyanions as chondroitin sulfate also produced a small, but definite rise in DNA (Tables 1 and 5). Restoration of DNA in DNA-ase nuclei is also confirmed by cytochemical data; after the action of DNA-ase the intensity of coloring of the nuclei with methyl green, according to Foelgen, is markedly weaker than in the intact nuclei; after incubation of the DNA-ase nuclei with the above mentioned polyanions, the DNA coloration of the nuclei becomes more intense [17].

In this connection we may believe that the restoration of the intensive protein synthesis in the DNA-ase nuclei after their incubation with DNA, RNA, chondroitin sulfate, or other polyanions [1-5] is connected with the ability of these compounds, in different ways, to restore the content of DNA in the nuclei.

It is of considerable interest to explain the question of the form in which the homologous, and especially the foreign DNA appears in the isolated nuclei: does it enter as a polymer or is it first split to an oligonucleotide; does it keep or lose its species specificity? For an answer to these questions we ran special experiments. The nuclei were further washed free of DNA-ase with 0.25 M sucrose and incubated with DNA. In the supernatant liquid after incubation we noted a great amount of acid soluble deoxyribonucleotides and not more than 20% acid insoluble unsplit DNA (Table 2); in a series of experiments the added polymeric DNA was completely split, and we found no acid insoluble fraction at all in the supernatant liquid. The addition to the incubation medium along with DNA of a

TABLE 2. Incubation of DNA with Nuclei under Conditions of Inhibition of Their DNA-ase Activity

Experimental conditions	Increase of DNA in nuclei, mg/ g	Amt. of deoxyribonucleotides in supernatant liquid, mg/g nuclei	Amt. of polymeric DNA in supernatant liquid, mg/g nuclei
Intact nuclei, incubated with DNA (20 mg / g nuclei)	–	–	20.1
DNA-ase nuclei, incubated with DNA (20 mg/g nuclei)	4.3	29.8	2.1
The same with citrate added to the medium (20 μmoles)	3.3	22.8	2.5
The same with added citrate and exclusion of Mg^{++} from the medium.	1.5	14.0	12.0

DNA-ase inhibitor, sodium citrate, very slightly lowered the ability of the nuclei to split the added DNA (the citrate which we added was enough to inhibit fully in vitro the action of all the DNA-ase which we usually added to the medium with the nuclei). Only when Mg^{++} was excluded from the incubation medium and sodium citrate was added did the DNA-ase activity undergo actual inhibition (though not complete); on this there increased the content of unsplit DNA in the supernatant liquid, and there was a decrease in amount of acid soluble deoxyribonucleotides (Table 2).

It was shown that under the conditions of our experiments intact nuclei, not treated with DNA-ase, do not have the ability to split DNA. Evidently nuclei submitted to the action of DNA-ase bind the enzyme tightly, and washing does not fully remove it. It is still not clear why the usual inhibitors prevent the activity of such DNA-ase, bound to the nuclei, weakly.

These results give us reason to suggest that the DNA added from outside, whether homologous or foreign, before it enters the DNA-ase nuclei, is split by them to mono- and oligonucleotides which evidently serve as materials for the resynthesis of their own DNA. This idea is also favored by the ability of the enzymatic hydrolyzate (dialyzate) alone to serve as the material for DNA resynthesis in the nuclei (Table 3). This is indicated by the fact that with inhibition of the splitting of externally added DNA (by removal of Mg^{++} and addition of citrate) the resynthesis of DNA in the DNA-ase nuclei is decreased (Table 2); evidently for the resynthesis of DNA in the nuclei we must have not externally added polymeric DNA, but the product of its enzymatic hydrolysis. It is natural to assume that foreign DNA (as well as homologous) during splitting loses its species specificity and is used only as material for resynthesis of the characteristic nuclear DNA; in this way, obviously, we cannot introduce new information into the nuclei which can serve as a basic "matrix" for the resynthesis of new, foreign proteins in the nuclei.

Not less important is an explanation of the problem of the path of resynthesis of DNA in DNA-ase nuclei on addition of RNA to them. As we already mentioned above, addition of RNA to the incubation medium stimulates resynthesis of protein in DNA-ase nuclei [2, 4]; Allfrey and co-workers considered that this effect was connected with transfer into the nuclei of polymeric RNA which in their opinion thus replaced the native depolymerized DNA. However, our experiments oppose this possibility since they show increased DNA in DNA-ase nuclei on addition of RNA to the medium; this was established by biochemical and cytochemical methods [16, 17]. The content of RNA in the nuclear fraction was also increased by this, but it accumulated, apparently outside the nucleus, especially in the pyroninophilic granules which we observed in the nuclear fraction after action of the DNA-ase [16]. All this gives us a basis for believing that the observed restoration of ability to synthesize protein in the nuclei on addition of RNA is also connected chiefly with the resynthesis of DNA in them.

We can think particularly of three different paths of participation of RNA in the resynthesis of DNA: 1) splitting of RNA to mono- and oligonucleotides which are transformed into deoxyribonucleotides and share in the resynthesis of characteristic nuclear DNA; 2) transformation of polymeric RNA in the nuclei into polymeric DNA (without preliminary splitting); 3) stimulation of DNA resynthesis in the nuclei on addition of RNA to the incubation medium because of a supply of nuclear deoxyribonucleotides.

TABLE 3. Effect of Addition of Enzymatic Hydrolyzates of Nucleic Acids on the Restoration of DNA in DNA-ase Nuclei

Experimental conditions	DNA content, mg/g nuclei	
	Expt. 4	Expt. 5
Intact nuclei	54.0	54.8
Nuclei incubated with DNA-ase (30 min, 37°)	24.7	23.4
Incubated DNA-ase nuclei with one of the additives in the substrate medium (60 min, 37°):		
DNA from calf thyroid	28.9	27.7
Enzymatic hydrolyzate of DNA (dialyzate)	28.7	–
Rat liver RNA	–	26.8
Enzymatic hydrolyzate of RNA (dialyzate)	–	26.1

TABLE 4. Incubation of RNA with Intact and DNA-ase Nuclei

Experimental conditions	Acid soluble ribonucleotide products in supernatant liquid, mg/g nuclei
Intact nuclei, incubated with RNA	1.7
Intact nuclei, incubated in a medium without RNA	1.06
DNA-ase nuclei, incubated with RNA	1.36
DNA-ase nuclei, incubated in a medium without RNA	0.9

Note: Acid soluble ribonucleotides in the rat liver, polymeric RNA itself [12], used as the substrate were less than 1% and did not appear on incubation of RNA alone in amounts of 0.5-5 mg in the medium.

The possibility of transformation of ribonucleotides into deoxyribonucleotides has been shown in recent years by a number of investigators [18-25]. Their results also favor this path of participation of RNA in DNA resynthesis. On incubation of intact nuclei with RNA, acid soluble products appear in the supernatant liquid with absorption maxima in the ultraviolet λ 260 mμ. Such substances were also found by us on incubation of RNA with DNA-ase nuclei. These acid soluble products contained ribose (Table 4).

It is known that Heppel and co-workers [26] found in guinea pig liver nuclei, an enzyme which split polyribonucleotides to 5 mono- and oligoribonucleotides. It is possible that in our experiments we met with the action on RNA of an analogous nuclear enzyme. We can assume that these products are used for transformation into deoxyribonucleotides and for later synthesis of DNA. It was shown that the enzymatic hydrolyzate of RNA can also restore the DNA content in DNA-ase nuclei (Table 3).

Recently a system was discovered which transformed ribonucleotides into deoxyribonucleotides; thus it was shown that cytidylic acid was converted into deoxycytidylic [27], and later, with the aid of deoxycytidylate deaminase,

TABLE 5. Increase of DNA in DNA-ase Nuclei When They Are Incubated with DNA RNA, or Chondroitin Sulfate

Experimental conditions	No. of expts (n)	Increase in DNA, mg, $M_d \pm m_d$	t_d
Incubation of DNA-ase nuclei with DNA	13	6.0 ± 0.17	35.0
Incubation of DNA-ase nuclei with RNA	13	2.9 ± 0.23	12.6
Incubation of DNA-ase nuclei with chondroitin sulfate	12	1.7 ± 0.35	4.9

Note: M_d mean difference; m_d difference in representative error; $t_d = M_d / m_d$, index of significance.

into deoxyuridylic [28] which in its turn, with the participation of thymidylate synthesase was methylated and converted into thymidylic acid [29]. In the isolated nuclei of calf thyroid there is uptake of ribo- and deoxyribonucleotides in the undialyzed acid insoluble fraction [30]. After uptake of cytidine triphosphate-C^{14} in this fraction, the acid soluble radioactive products were liberated from it by the action of DNA-ase, and treatment with RNA-ase and alkaline hydrolysis did not result in accumulation of such products. Evidently it is a question of uptake of CTP-C^{14} in DNA; however, in what form the ribonucleotide is taken up by the DNA of isolated nuclei is still not clear from this work.

These experiments and the literature data contradict the idea of direct conversion of polymeric RNA into polymeric DNA; they rather indicate the participation in the DNA resynthesis of products of RNA depolymerization.

We have already spoken above of the ability of chondroitin sulfate to stimulate the resynthesis of DNA in DNA-ase nuclei (Table 1). Cytochemical reactions (for polysaccharides and DNA) do not appear in DNA-ase nuclei from chondroitin sulfate after its incubation with this substrate, but increase in DNA is observed [15]. It is possible that this reaction is not sufficiently sensitive to detect chondroitin sulfate in the nuclei. Biochemical determination shows the accumulation of about 20% of the added chondroitin sulfate in the nuclear fraction after incubation with it, but it is still not clear whether it is in the nuclei or outside them (for example, in the pyroninophilic granules of the nuclear fraction which we previously described [16, 17]]. The observed resynthesis of DNA in the nuclei when chondroitin sulfate is added still cannot exclude the increase of this polyanion itself in the nuclei. We still cannot explain satisfactorily the role of chondroitin sulfate in DNA- resynthesis; in this case it is limited to the idea of a stimulating effect of this polyanion on DNA synthesis perhaps because of an intranuclear base of deoxyribonucleotides of the DNA-ase nuclei. If such a mechanism exists, we also cannot exclude the full possibility of a similar participation of polymeric RNA in DNA resynthesis (RNA on incubation with the nuclei is only partly split), although this seems less probable.

Comparison of the effects of externally added DNA, RNA, and chondroitin sulfate on the intensity of DNA synthesis in the nuclei shows their important differences in this respect (Table 5). The most marked resynthesis of DNA in DNA-ase nuclei is produced by the addition of homologous or foreign DNA to the incubation medium; it is less marked with addition of RNA and least with addition of chondroitin sulfate. However, we note that even in the case of greatest resynthesis there is restoration of only a small part of the DNA destroyed by the DNA-ase in the nuclei.

The DNA content in 1 g of nuclei in our experiments varied from 30 to 50 mg, averaging 40 mg (this value was determined by the conditions of isolating the nuclei, density of the precipitate in centrifuging, and other factors; we did not carry out calculation of DNA content in one nucleus). After the action of DNA-ase the DNA content fell on the average by 60% and was 15 mg/g of nuclei. As Table 5 shows, after addition of DNA to the incubation medium, its increase in the DNA-ase nuclei on the average was 6.0 mg/g of nuclei; when RNA was added, 2.9 mg/g of nuclei, and with chondroitin sulfate, 1.7 mg/g of nuclei. Statistical analysis showed a high degree of significance ($p > 0.999$) for the increase of DNA in each group. With respect to the average DNA content in the DNA-ase nuclei

which we found (15 mg/g of nuclei) this increase with addition of DNA was 40%, with RNA, 19.5%, and with chondroitin sulfate, 11.5%; however, with respect to the total amount of DNA in the intact nuclei (40 mg/g of nuclei) the increase was 15, 7, and 4%, respectively. In the experiments of Allfrey and co-workers [4, 31], 71-77% of the nuclear DNA was destroyed by the action of DNA-ase, and the restoration with addition of DNA to the medium was 13-14%, an increase very close to that which we found. The synthesis of proteins in the nuclei fell correspondingly by 46-84% and the restoration after addition of DNA to the DNA-ase nuclei was 27-52%. Other functions which had been destroyed in the partial depolymerization of DNA in the nuclei were also restored here, the synthesis of ATP and uptake of precursors into RNA. Thus, the relatively small (13-14%) increase in DNA nevertheless assured a marked restoration of protein synthesis and other processes in the nuclei. In this connection it is possible that there is a definite degree of restoration of these processes from the increase in DNA found in our experiments on addition of RNA and chondroitin sulfate (respectively 7 and 4%).

At the present time it has already been shown quite clearly by a number of investigators that the DNA in the nuclei is not homogeneous [32-37] and can be separated into a number of fractions which differ in physical and chemical properties and are probably also unequal in their biological activity. We can assume that the synthesis of proteins is chiefly connected with some one of the DNA fractions which is first restored in the external addition of DNA, RNA, and chondroitin sulfate. It is possible that with respect to percentage this makes up a relatively small part of all the nuclear DNA. With this idea we could explain the contradiction which we observed: a relatively small resynthesis of DNA and a marked restoration of the ability to synthesize proteins in the nuclei. We have studied the composition of the DNA fractions in intact, DNA-ase, and restored nuclei.

The data from this work indicate the participation of the products of enzymatic splitting of DNA in its resynthesis. We can assume that this process is assured by the undestroyed DNA which remains in the nucleus and perhaps plays the role of a "primer." In the well known work of Kornberg and co-workers [39, 40] it was shown that such a "primer" determines the nucleotide composition of DNA synthesized by polymerase from deoxyribonucleotide triphosphates. Among the products of enzymatic hydrolysis of DNA, which have been shown in our experiments to be the chief materials for DNA resynthesis, the oligonucleotides predominate and the content of mononucleotides does not exceed 1% [41]. The possibility has been shown of participation of oligonucleotides in DNA synthesis under the influence of calf thymus polymerase [42]. Belousova [21] has shown the possibility of using oligonucleotides in DNA synthesis in cancer tissue.

SUMMARY

Under the action of DNA-ase isolated cell nuclei from calf thyroids lose 50-80% of the DNA. On addition to the medium with such nuclei of homologous or foreign DNA, it is split by 80-100% to acid soluble oligonucleotides and the DNA content in the nuclei is then restored by an average of 15%. Restoration of the DNA in the nuclei also occurs on addition to the incubation medium of the dialyzate from the products of enzymatic hydrolysis of DNA. It is suggested that the DNA added to the "DNA-ase" nuclei is split by the residual DNA-ase bound to the nuclei, and restoratiion thus occurs because of the products of its splitting. Intact nuclei do not split added DNA. On addition of RNA to the medium with DNA-ase nuclei, an increase in DNA is also noted. It is shown that intact and DNA-ase nuclei have the ability to split added RNA to acid soluble ribonucleotide products which perhaps can be converted to deoxyribonucleotides and which can serve as materials for resynthesis of DNA in the nuclei. The addition of chondroitin sulfate to the medium with DNA-ase nuclei causes a small, but significant increase of DNA in the nuclei. It is suggested that the DNA resynthesis in this case can occur because of a reserve of nuclear deoxyribonucleotides.

The authors suggest that the restoration of the ability of the nuclei to synthesize proteins, which was found by previous investigators [1-5], on addition of homologous or foreign DNA, RNA, or chondroitin sulfate to the DNA nuclei occurs as a result of the resynthesis in such nuclei of their characteristic DNA.

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THE ISOLATION FROM HORSE ERYTHROCYTES OF CRYSTALLINE CATALASE AND A STUDY OF SOME OF ITS PHYSICOCHEMICAL PROPERTIES

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Although catalase is widely distributed in living organisms, its biological significance has not been studied up to now. In recent years communications have appeared which show the great role of catalase in oxidation-reduction processes of the animal cell [1-7]. It is interesting to see that catalase does not decompose the rather slight concentration of hydrogen peroxide which can occur in tissue metabolism.

In the present work the authors have set the problem of improving the process of isolation of crystalline catalase from horse erythrocytes, a waste product in serum production, and a study of some physicochemical properties of this preparation.

As the basis of obtaining crystalline catalase we have taken the method worked out by Laskowski and Sumner [8]. These authors isolated crystalline catalase from beef erythrocytes. In obtaining catalase a very important step is its separation from hemoglobin. We succeeded in finding the best ratio of ethanol and chloroform, at which hemoglobin was comparatively easily separated from solution. Also, to obtain a good water soluble preparation of catalase we recrystallized it in a phosphate buffer. On this there was removal of mucin like protein substances which make the process of crystallization of this enzyme especially difficult.

Method of Isolating Crystalline Catalase

To 5 liters of fresh horse erythrocytes was added an equal volume of distilled water and the mixture was left in a refrigerator room at 4° for 8-10 hr for hemolysis of the erythrocytes. To the resulting hemolyzate we added an equal volume of 96% ethanol and chloroform calculated at 100 ml per liter of solution, to remove hemoglobin. The mixture was energetically stirred for 15 min, the hemoglobin precipitate was removed, and the cloudy solution was filtered through a pleated filter paper. To the resulting light brown filtrate was added an equal volume of ethanol and the mixture was left for 12-16 hr at 2°. The gray precipitate was filtered on a paper filter, scraped off, and treated with 1 M phosphate buffer, pH 7.4, calculated at 2 ml of buffer per 1 g of precipitate; the insoluble part was filtered off. The resulting dark brown filtrate was treated with a small amount of infusorial earth, after which it was again filtered through a pleated filter. To the filtrate, brought by addition of 1 N H_2SO_4 to pH 5.4, was added with careful stirring a saturated solution of $(NH_4)_2SO_4$ to a light cloudiness, and it was left for a day at 4° The precipitate was filtered off and dried in a vacuum desiccator at 0°. The resulting powder, black in color, weighed 1.5 g and was a preparation of amorphous catalase.

To obtain crystalline catalase the precipitate was dissolved in phosphate buffer, pH 7.4. The insoluble part was filtered off and the resulting filtrate was acidified with 1 N H_2SO_4 to pH 5.4. The resulting cloudy liquid was centrifuged at 5000 rpm. To the clear centrifugate was added a saturated solution of $(NH_4)_2SO_4$ to slight cloudiness. After standing for 24 hr at 0° a needle shaped precipitate came down and was filtered off and dried. The yield of crystalline catalase was 100 mg/liter of erythrocytes.

At all stages of isolation of catalase we determined the catalase activity in precipitates and filtrates (Cat. A) by the classical method of Euler and Josephson [9]. According to this method we first find the reaction constant (K) of the first order decomposition of H_2O_2 for a definite time interval by the formula:

$$K = 2{,}3\frac{1}{t} \cdot \lg \frac{x_0}{x_1} \ (x_2,\ x_3 \text{ etc.}),\quad),$$

where x_0 is amount of substrate at initiation of the reaction, x_1, x_2, x_3, etc. the amounts of substrate after different time intervals from the start of the reaction (3, 6, 9, etc. min).

By extrapolation of the curve we obtain K_0, the reaction constant of the first order for zero time. Cat. A. is calculated by the formula:

$$\text{Cat. A} = \frac{K_0}{A},$$

where A is the content of enzyme in grams in the experimental sample consisting of 1/150 M phosphate buffer (pH 6.8), 0.01 N H_2O_2, and the enzyme solution. The total volume of liquid should always be constant; in our case it was 50 ml.

As the catalase was freed from proteins, mucous substances, and pigments, the catalase activity rose markedly. The hemolyzed erythrocytes had Cat. A 272. After the first precipitation of protein and other substances from the hemolyzate with 96% alcohol and chloroform, the Cat. A. of the filtrate was 1748. Cat. A of the crude precipitate on the filter after $(NH_4)_2SO_4$ precipitation was 10023, and the catalase activity of the crystalline preparation was from 24,000 to 40,000.

When the crystalline and amorphous forms of the enzyme were kept, their activity decreased. It should be noted here that after the first month the activity of the preparations fell by 20-30%, and in later months, slightly, but practically remained constant. The dry enzyme preparation kept best of all in a hermetically sealed tare in the dark at 0°. Very dilute solutions of catalase were unstable when kept.

Characteristics of the Isolated Preparation

The thus obtained preparation of crystalline catalase with Cat. A 30,000 was studied physicochemically. Chemical analysis showed that it contained 70% protein. The amount of iron, determined by the o-phenanthroline method, was 0.1%. When a 2% catalase solution in phosphate buffer, pH 7.8, was submitted to the ultracentrafuge (50,000 rpm), three peaks were observed. The largest fraction, consisting of $\simeq$ 70% catalase, had sedimentation constant $S_{20,w}$ equal to 11.4 S. For the two other fractions, which made up 15% of the preparation each, the corresponding values were 7.54 and 3.16 S. The mol. wt., of the crystalline catalase ($S_{20,w}$ 11.4 S) was 225 000.* Similar results were obtained by other authors [10-12].

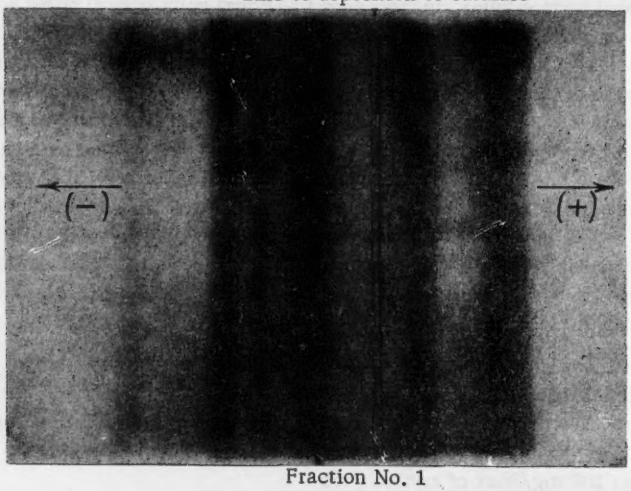

Fig. 1. Electrophoretogram of catalase separated into fractions in agar-agar.

*The ultracentrafuge work was carried out by S. Ya. Frenkel to whom we express thanks.

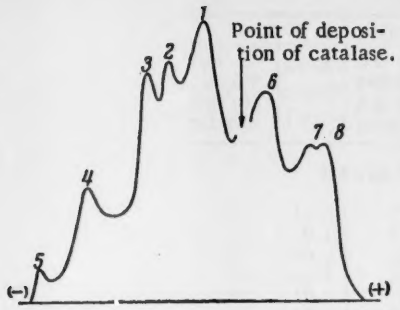

Fig. 2. Densitogram of electrophoretogram of catalase in agar-agar, obtained on a recording microphotometer MF-4. From 1-8 number of fractions.

The crystalline catalase was further submitted to electrophoretic study on agar [13]. Electrophoresis was carried out for eight hours in a veronal buffer, pH 8.6, μ 0.05, at 220 v and current strength 45-50 ma. Fixation was carried out in 5% TCA for 12 hr, after which the TCA was washed out with distilled water (24 hr) and the agar plates were dried in a thermostat at 37°. Staining was produced with 0.02% solution of water soluble aniline blue for 3-4 hr. The excess dye was washed out (30-60 min) with distilled water (Fig. 1). In Fig. 2 we give the densitogram of the electrophoretogram for catalase on agar, obtained with a recording microphotometer MF-4. From Figs. 1 and 2 we see that under our experimental conditions the preparation of crystalline catalase is separated into eight fractions. Five fractions move to the cathode and three to the anode. In each fraction we determined the amount of protein, iron, and catalase activity, which was calculated per mg of protein. The results of the analyses showed that 90% of the total amount of iron in the preparation was contained in the first fraction, which moved to the cathode at the lowest rate. This fraction had the maximum catalase activity. In the second and third fractions, which moved more rapidly to the cathode, the activity was much less, and the fourth and fifth had none at all. No activity was noted in any of the fractions which moved to the anode. Hence, our crystalline catalase had essentially three active fractions. Samajimu [12] came to similar conclusions in the separation of catalase on an ion exchange resin.

SUMMARY

In the modification which we have suggested for obtaining crystalline catalase from erythrocytes, hemoglobin is removed with an ethanol-chloroform mixture and precipitation of catalase is carried out in a phosphate buffer. A catalase easily soluble in water is thus obtained. In the catalase preparation with activity 30,000 there is 0.1% iron. Its molecular weight is 225,000. In electrophoresis on agar there are three active fractions which move to the cathode. The most active fraction moves to the cathode at the lowest rate. In ultracentrafuging there are three peaks with sedimentation constants ($S_{20,w}$) equal to 11.4, 7.54, and 3.16 S.

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THE PARTICIPATION OF THE NERVOUS SYSTEM IN REGULATION OF THE ACTIVITY OF GLUCOKINASE IN MUSCLE

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Glucokinase is a "tight" enzyme link which determines the rate of the process of glycolysis as a whole, in this case, when the substrate is glucose [1]. Therefore the question of the regulation of the activity of glucokinase is especially important [2].

Cori and co-workers observed the inhibiting effect of extracts of the anterior lobe of the pituitary and of the adrenal cortex on the activity of muscle glucokinase, and also the action of insulin in preventing the inhibition [3-5]. The results of the experiments of Cori were not confirmed when they were repeated by Stadie [6]. However, Bacila and Barron showed an inhibition of glucokinase activity in the hyaloplasm, from muscle, after addition of some corticosteroid hormones [7]. The activity of glucokinase of muscle extracts was also inhibited by β-lipoproteins isolated from the blood plasma of diabetic humans and rats with alloxan diabetes [8]. Experiments on eviscerated animals [9] and on isolated rat diaphragms [10] showed that the permeability of intact muscle cells for glucose increased under the action of insulin. Independent of the mechanism of action of insulin, its stimulating effect on the rate of the glucokinase reaction is undoubted.

At the present time there are no results on the participation of the nervous system in regulation of the glucokinase reaction in muscle. Humoller in rats [11] and Gerritsen in rabbits [12] noted a sharp rise in activity of glucokinase in muscle extracts made atrophic after denervation. In the opinion of Gerritsen this fact depends on the unequal splitting of some muscle proteins in the atrophy (a decrease in content of contractile proteins while preserving some of the enzymes, particularly glucokinase); the increase in activity of glucokinase in denervated muscle is thus relative. However, we cannot exclude the possibility that the destruction, by innervation, of the muscle may in itself play a part in increased activity of the enzyme. If this suggestion is confirmed, the question arises of the possibility of participation of the nervous system in the regulation of the rate of the glucokinase reaction, and in connection with this, also the rate of glycolysis in the muscle.

Schonfelder studied the glycolytic activity of muscles of newborn animals and denervated muscles of adult animals and found that addition of glucose increased the formation of lactic acid in both [13]. However, these results were not confirmed by later workers [14]. According to the idea of the "tight link" in glycolysis [1], the increased activity of glucokinase should be accompanied by an increased formation of lactic acid from glucose which actually occurred in the experiments of Kazakova in cancer cells [15] and of Agol, Gershanovich, and Étingof in cultures of monkey kidney cells [16].

There is well known interest in study of the interaction of the nervous system and hormones in their regulatory effects on the activity of glucokinase.

The present investigation is intended to check the reality of the ideas expressed above. First we ran experiments on study of the activity of muscle hexokinases: glucokinase and fructokinase in extracts from muscles atrophied after denervation, and from atrophied muscles with retained innervation (tenotomy).

METHODS

The experiments were run on rabbits weighing 2.0-3.0 kg. Denervation of the gastrocnemius muscle was carried out by direct section of a portion (1 cm) of the sciatic nerve; tenotomy, by section of part of the Achilles

TABLE 1. Glucokinase Activity of Denervated and Tenotomized Muscle

Control		Experimental		Loss in wt. of operated muscle, %	Control		Experimental		Loss in wt. of operated muscle, %
loss of glucose, μg/mg protein	wt.of muscle (g)	loss of glucose, μg/ mg protein	wt.of muscle (g)		loss of glucose, μg/ mg protein	wt.of mus-cle, g	loss of glucose, μg/ mg protein	wt.of mus-cle, g	
Denervated					Tenotomized				
27	11,9	57	6,6	45	40	7,3	42	4,8	35
31	9,1	64	5,6	39	37	11,1	28	7,2	35
32	10,9	45	6,0	45	32	9,7	33	5,5	43
31	12,1	64	6,7	45	25	11,5	25	6,6	42
39	10,0	68	4,9	51	11	12,1	12	8,0	34
34	12,7	84	7,0	45	29	4,6	28	2,5	46
11	20,1	40	9,6	53	15	15,9	14	7,7	52
23	5,5	41	4,8	13	16	11,4	16	6,9	40
14	11,1	26	7,6	32	10	13,0	8	7,9	40
17	13,2	44	7,4	44	16	19,0	16	9,7	49
21	8,3	62	5,3	37	17	20,0	13	11,3	44
27	17,0	49	9,0	47	25	11,1	19	5,6	50
24	10,0	114	3,8	62	18	12,3	18	8,0	35
26	10,9	70	5,4	51					

tendon. The gastrocnemius muscle of the other leg served as the control. The animal was placed in the experiment usually 3-4 weeks after the operation. After this period the animal was decapitated, and the gastrocnemius muscle was quickly weighed, cooled with ice, and cut up with scissors. We added 2 ml of 0.15 M KCl per 1 g of wet weight of the mince and ground the mixture with quartz sand for 5-10 min. After an hour the extract was separated by centrifuging at 600 g for 5 min.

The glucokinase activity was determined by decrease in glucose after 15 min incubation of the extract at 37°. The incubation mixture contained [17]: extract, 0.15 ml; glucose, 0.1 ml, 0.0028 M; K-ATP, 0.1 ml, 0.005 M; phosphate buffer, pH 7.9-8.0, 0.4 ml, 0.042 M; $MgCl_2$, 0.1 ml, 0.005 M; NaF, 0.1 ml, 0.05 M; KCl, 0.05 ml, 0.03 M (in all cases the final concentration).

The glucose content in the extract was determined by the method of Hagedorn-Jensen with precipitation of the protein by $CdSO_4$-NaOH [18]. Protein was determined by the biuret method [19]. The activity of fructokinase was determined by decrease in fructose after 20 min incubation at 37°. The incubation mixture had the same composition as in determining the activity of the glucokinase except that it did not contain NaF and had a different pH (7.3-7.4, the optimum for fructokinase). Fructose was determined after precipitation of the protein with $CdSO_4$-NaOH in the protein-free filtrate by the method of Roe [20].

In the experiments on the study of glycolysis we measured the increase in lactic acid after 30 min incubation of the extract at 37°. Lactic acid was determined in the TCA filtrate by the colorimetric method [21]. In the experiments using glucose as the substrate for glycolysis we determined the activity of the glucokinase in parallel. If we used glycogen as the substrate (final concentration 0.4%) incubation was carried out at pH 7.2-7.3.

In the experiments with addition of cortisone to the extract we added 100 μg of cortisone acetate in the experimental sample. Both extracts, experimental and control, were kept after addition of the hormone until the beginning of incubation at 0° for 30 min. Cortisone acetate was added to the extract according to the description of Bacilia and Barron [7].

RESULTS

Activity of Hexokinases in Denervated and Tentomized Muscle

The results of the experiments confirmed the literature data [11, 12] on the increased glucokinase activity of denervated muscle (Table 1). In all 14 experiments there was a marked increase in fall of glucose in extracts from denervated muscle as compared to the controls, on the average by 134%, that is, more than double. Both in the experiments with denervation and those with tenotomy there was about the same fall in weight of the atrophied gastrocnemius muscle (on the average, by 44% in denervation and 43% in tenotomy). We should mention that in

TABLE 2. Fructokinase Activity of Denervated and Tenotomized Muscle

Control		Experimental		Loss in weight of operated muscle
loss of fructose, μg/mg protein	weight of muscle, g	loss of fructose, μg/g protein	weight of muscle, g	
		Denervated		
13	10.0	35	5.0	50
12	12.4	36	7.3	40
9	11.1	43	5.1	54
0	9.8	49	4.9	50
7	10.2	31	5.5	46
9	10.2	27	6.0	48
		Tenotomized		
12	9.4	11	4.4	53
4	11.0	5	5.7	48
13	10.5	10	5.4	49
12	12.3	11	6.0	51

atrophy caused by tenotomy there is destruction of the contractile protein of the muscle. However, an increase in glucokinase activity in the extracts from tenotomized muscle as compared to the control was not found (Table 1).

Comparing the results of the experiments on measuring the glucokinase activity in extracts from denervated and tenotomized muscles permits the conclusion that the increase in glucokinase activity in muscle, atrophied after denervation, basically depends on the cessation of the entry of nervous impulses in the muscle. We ran parallel experiments on determination of the activity of the second hexokinase of muscle, fructokinase, first discovered by Cori and co-workers [22].

The results of the experiments showed that denervation leads to a sharp increase in activity of fructokinase also (Table 2). The activity of fructokinase of muscle extracts 3-4 weeks after denervation of the muscle reached a value close to the activity of glucokinase of normal muscle, or even higher values (control, Table 1). At the same time in the extracts from tenotomized muscle the activity of the fructokinase, like that of glucokinase, remained at the level of the control.

Since the role and significance of fructokinase and the fructokinase reaction in muscle metabolism is still unclear, further experiments were devoted to a study of the significance and conditions for increasing the activity of glucokinase in denervated muscle.

Glycolysis in Extracts of Denervated Muscle

The results of experiments on measuring glycolytic activity of muscle extracts correspond fully with the ideas on relation of rate of glycolysis to activity of glucokinase.

In all the experiments (Table 3) the enzyme activity in the denervated muscle exceeded the control by 183%, and the level of glycolysis (using glucose as the substrate for glycolysis) by 105%. In the experiments with glycogen the increase in lactic acid in the extracts from denervated muscle was markedly lower than in the control. This fall in glycolysis agreed with the literature data on the significant lowering of activity of phosphorylase in denervated muscle [11, 23]. We should remark on the similarity with the results of experiments in cancer cells, in which a high rate of glycolysis (substrate glucose) is connected with a high activity of glucokinase [15, 24], and a low rate of glycolysis (substrate glycogen) with a lower phosphorylase activity.

TABLE 3. Glycosis in Extracts from Denervated Muscle Increase and Decrease of Lactic Acid Expressed in μg/mg Protein After 30 min

Control		Experimental	
increase in lactic acid	fall in glucose	increase in lactic acid	fall in glucose
Substrate, glucose			
67	42	125	140
60	54	147	111
60	45	165	148
50	46	100	121
57	35	76	101
50	33	100	124
Substrate, glycogen			
300	–	157	–
317	–	126	–
407	–	227	–
415	–	264	–
210	–	102	–

TABLE 4. Glucokinase Activity in Muscles of Alloxan Diabetic Rabbits

Blood sugar, mg %	Loss of glucose in μg/mg protein after 15 min	
	control muscles	denervated muscles
280	34	31
400	53	59
355	24	35
365	32	49
320	33	75
320	47	50
300	30	72
312	77	67
329	15	35
–	40	66
–	48	56
247	31	49
300	33	39
–	17	24

Hormonal Regulation of Glucokinase Activity in Denervated Muscle

The hormonal regulation of the rate of the glucokinase reaction under conditions of a denervated muscle is studied according to the well known ideas of Kenyon [25] on the increased sensitivity of denervated structures to various hormonal and pharmacological agents (law of denervation). It is assumed that one of the reasons for the high activity of glucokinase in denervated muscle may be the increase in the sensitivity to stimulation of the glucokinase reaction by the action of insulin. If this idea is correct, we can expect that decrease in production of insulin by the β-cells of the island apparatus of the pancreas (in starvation, and to a greater extent in diabetes) may cause a lowering of glucokinase activity in denervated muscle, perhaps to the level of the control muscle.

TABLE 5. Effect of Cortisone on Glucokinase Activity of Normal Tenotomized and Denervated Muscles (loss of glucose expressed in μg/mg protein)

Control (normal muscle)		Experimental		Control (normal muscle)		Experimental	
without cortisone	100 μg cortisone	without cortisone	100 μg cortisone	without cortisone	100 μg cortisone	without cortisone	100 μg cortisone
Tenotomized				Denervated 3-4 weeks before expt.			
21	17	37	31	17	11	38	44
14	13	22	20	16	12	39	47
20	18	19	19	9	6	45	46
19	17	34	29	17	14	70	73
28	28	29	26	22	16	61	64
12	12	20	17	27	23	36	38
18	16	29	20				
30	25	31	24	Denervated 3 days before expt.			
35	31	39	27				
21	15	22	18	32	26	30	29
19	13	24	15	52	43	57	55
				23	15	22	23
				18	16	15	17
				20	18	23	25
				24	18	16	17

We ran two sets of experiments on animals with insulin deficiencies. For the first series we used starved rabbits. It is known that in starvation the secretion of insulin is decreased because of atrophy of the Islands of Langerhans. The activity of liver glucokinase in starvation is greatly lowered [26].

In the experiments of the first series we carried out denervation of the gastrocnemius muscle of rabbits, and beginning on the 18th day after the operation, we deprived the animals of food, giving them only water. After 7-10 days the rabbits lost 25-35% of their original weight. Determination of loss in glucose (in μg/mg protein) in samples of extracts from control and denervated muscles of these animals gave the following:

Control muscles	Denervated muscles	Control muscles	Denervated muscles
26	38	9	13
11	16	11	29
9	39	13	24
5	13	8	29

As these results show, decreased glucokinase activity in starvation occurs proportionally in the control and in the denervated muscles, and so the difference between them remains as sharp as in the normally fed animals. It is possible that the absence of the expected "leveling" of enzyme activity in the control and denervated muscles depends on the relatively small degree of insulin deficiency in the starved rabbits.

Therefore a second series of experiments was run on animals with alloxan diabetes. Just as in the experiments with starved subjects, we denervated and on the 18th to 20th day after the operation, that is, when the glucokinase activity had already increased, the rabbits were injected intravenously with 150 mg/kg of alloxan. After 10-12 days from the injection, at the height of development of the diabetes (at blood sugar not less than 250 mg%), we measured the glucokinase activity of the muscle extracts (Table 4).

The results of this series of experiments varied. Of 14 experiments, in three there was the same rise in glucokinase activity as in healthy animals; in six cases the activity increased only one half; in five experiments no increase at all was found. Since at the time of injecting the alloxan, that is, 18-20 days after denervation of the muscle, the glucokinase activity in it was already increased (Table 1), then we can consider on the basis of these results that the increased glucokinase in denervated muscle is reversible. The variation in the results probably depends on different degrees of diabetes caused by alloxan in different animals. The relative decrease in glucokinase

activity in the denervated muscles of animals with insulin deficiencies confirms to a marked extent the ideas expressed above. Evidently the cessation of entry of nervous impulses into the muscle gradually leads to a shift in the equilibrium in the action of hormones on glucokinase activity and to a dominance of the effect of insulin on this enzyme.

Such a shift in equilibrium can be reflected not only in increased sensitivity of the muscle to the action of insulin, but also to a lowered sensitivity to inhibition of hormonal effects, for example, to cortisone. Inhibition of glucokinase in muscle extracts by addition, in vitro, of cortisone was shown in 1954 by Bacila and Barron [7]. We ran experiments to study the effect of cortisone on glucokinase activity of extracts from normal, denervated, and tenotomized muscles (Table 5).

The results of these experiments show that addition of cortisone inhibits glucokinase activity only in extracts from muscles with retained innervation (normal and tenotomized). Cortisone does not inhibit glucokinase activity in extracts from denervated muscles independent of the degree of their atrophy (3 weeks or 3 days after denervation).

DISCUSSION

On the basis of these studies we can conclude that the rate of the glucokinase reaction in muscles depends on a regulating effect of the nervous system. Evidently the entry of nervous impulses into the muscle has significance for preserving a definite equilibrium in the hormonal regulation of the rate of the glucokinase reaction. Destruction of the connection of the muscle with the nervous system leads to a shift in this equilibrium toward a dominating effect of insulin. It seems probable that one of the reasons for increased glucokinase activity in denervated muscle is just this change in its reactivity to hormones: increased sensitivity to insulin and decreased sensitivity to the inhibiting action of cortisone. We cannot, however, exclude the possibility of direct participation of the nervous system in regulating the glucokinase activity.

Denervation leads to change in the process of glycolysis in the muscle. In accord with the literature [14, 23] there is lessened utilization of glycogen. The use of glucose as substrate, on the other hand, increases. Although this conclusion contradicts the results of Épel'baum and Kantor [14] it confirms the earlier data of Schönfelder [13] on the increased utilization of glucose by denervated muscles and muscles of newborn animals. These results, in accord with the data of Haarman [27] on the similar increase in utilization of glucose by smooth muscle, agree with the well known ideas of Orbeli on the reversion of denervated muscles to a more primitive form of musculature [28].

It is interesting to note that high glucokinase activity, and the related portions of glucose as the substrate, used by the tissues for formation of lactic acid, are all characteristics of those tissues in which nervous regulation is insufficiently developed, destroyed, or is absent: in embryonic and smooth muscle [13, 27], in denervated muscle [11-13], in malignant tumors [15, 24], and in cultures of kidney tissue cells [16]. For denervated muscles and malignant tumors the absence of inhibiting action on glucokinase activity by added cortisone is also characteristic [7].

SUMMARY

In extracts from rabbit gastrocnemius muscles, atrophied after denervation after 3-4 weeks, there is a sharp rise in glucokinase activity (2-2.5 times) and in fructokinase activity (4 times). In extracts from muscles, atrophied after tenotomy with the same degree of fall in weight of the atrophied muscle as in the denervated ones, the glucokinase and fructokinase activity is not changed.

Increased activity of muscle hexokinases, in general, depends on the cessation of entry of nervous impulses into the muscle.

The glycolytic acitivity of the extracts from denervated muscles decreases when the substrate for glycolysis is glycogen, and increases when glucose is used as the substrate.

Starvation leads to a proportional fall in glucokinase activity in both denervated and control muscles. Here the enzymatic activity of the denervated muscle remains twice as high as the activity of the control muscle.

Alloxan diabetes leads to a relative decrease of glucokinase activity in denervated muscle, while the activity of the enzyme in the control muscle not only is not decreased, but is even somewhat above the enzyme activity of the muscles of healthy animals.

Cortisone, added in vitro, hinders glucokinase activity in extracts of muscles with retained innervation, either normal or tenotomized. Cortisone does not inhibit glucokinase activity in extracts from denervated muscles, independent of the degree of their atrophy.

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GLYCOPEPTIDES LIBERATED FROM EGG ALBUMIN BY PROTEASES

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In seeking enzymes which can split polysaccharide complexes from egg albumin we have studied the action of various proteases on this protein. Enzymatic hydrolysis was carried out by trypsin alone or by a combination of enzymes, trypsin-chymotrypsin, pepsin-trypsin-chymotrypsin, and, finally, by the whole complex of enzymes in pancreatic juice, directly or after the action of pepsin. None of these combinations of enzymes liberated polysaccharides, as follows from the fact that we isolated from the hydrolyzates glycopeptides which contained 80-90% of the carbohydrates of the whole protein. At the same time, no free polysaccharides were obtained.

Depending on the enzyme used for splitting, the peptides of these glycopeptides were of different lengths and composition. We determined the qualitative and in some cases the quantitative amino acid composition of the glycopeptides which we isolated. This permitted us to mark out from comparison of the peptides compositions of different lengths the approximate order of arrangement of the amino acids in the peptide chain of egg albumin, which are directly joined to polysaccharides.

METHODS

Preparation of enzymatic hydrolyzates of egg albumin and isolation of the glycopeptides. The preparation of egg albumin was recrystallized six times and after removal of salt by dialysis, was dried lyophilically. The content of nitrogen was 15.5%, of carbohydrates, 1.80%.

For enzymatic splitting we prepared a 2% solution of the protein in 0.1 N HCl for pepsin hydrolysis and in 0.01 N HCl for hydrolysis by other enzymes. We later made it alkaline with $NaHCO_3$ to a pH of about 8 after denaturation by boiling.

The addition of enzymes was carried out in several successive portions in the course of the first 3-7 days, in all 1-2% of the weight of the protein. Hydrolysis by pepsin lasted 2 days, by the other enzymes 15 days and in special experiments up to 30 days at 37°. The solutions at the time of hydrolysis were kept under toluene. At the end of hydrolysis the enzymes were inactivated by heating, the insoluble precipitate was filtered off, the solution was evaporated in a vacuum to low volume, and was precipitated at pH 2 with four volumes of acetone. The glycopeptides were separated from the precipitate by chromatography on a column with Zeocarb-225 (H^+) and separated by electrophoresis at pH 2.7 into fractions. The methods were described in detail in a previous work [1].

The yield of carbohydrates in the glycopeptides after their separation on the column was 80-90% of their starting content in the protein. For the trypsin hydrolyzate only, they were 42%, which is due to the presence in these glycopeptides of basic amino acids which permit a stable binding of glycopeptides on the cationite and make their extraction by water difficult.

Characteristics and analysis of the glycopeptides. The isolated glycopeptides when dried in a vacuum desiccator formed a clear film, easily soluble in water. In paper chromatography in different solvent systems: [butanol-acetic acid-water (4 : 1 : 5), pyridine-isoamyl alcohol-water (10 : 10 : 7), pyridine-ethyl acetate-water (1 : 2 : 2)] they did not move from the line of deposition. Glycopeptides gave a positive reaction for nitrogen with tolidine after chlorination, according to Reidel and Hoppe [2], and for glycol groups with benzidine, after oxidation with HIO_4 [3]. The reaction for free reducing groups with aniline hydronaphthalate and with $AgNO_3$ was negative.

In alkaline and neutral medium the glycopeptides in paper electrophoresis move into one zone, but at pH 2.7 there is clear separation into 3-4 zones. Electrophoresis was carried out in 1.5% acetic acid at 500 v for 10 hr in a

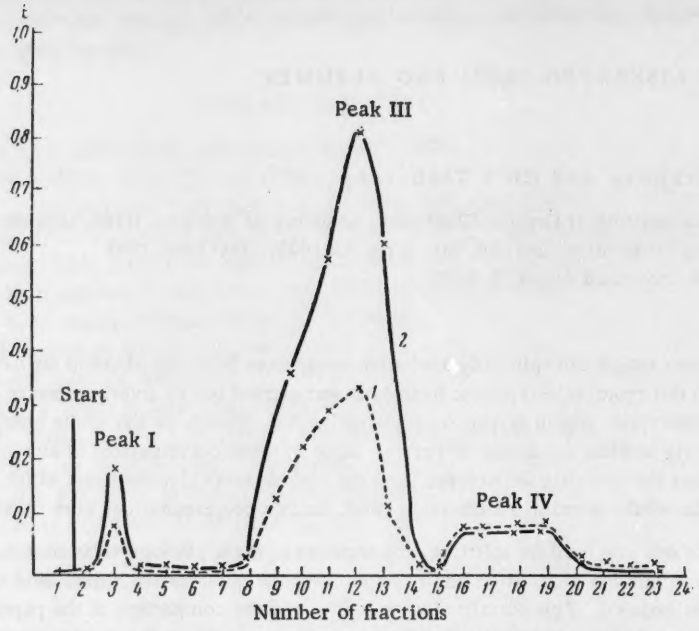

Fig. 1. Electrophoretic separation of glycopeptides obtained after action of pepsin, trypsin, and chymotrypsin, on a cellulose block. 1) Content of glycopeptides by ninhydrin reaction; 2) content of carbohydrates by reaction with orcin.

cold chamber. For separation on a cellulose mass the block, after electrophoresis was cut into cross sections 1 cm wide and each portion was extracted with water. The content of peptides in the extracts was determined colorimetrically by the ninhydrin reaction [4], and the carbohydrate content by orcin [5].

In Fig. 1 we give typical curves for separation of the hydrolyzate obtained after successive action of pepsin and a mixture of trypsin and chymotrypsin into three zones. In the case of hydrolysis by pancreatic juice or supplementary action of carboxypeptidase still another zone was formed (2) between zones 1 and 3, which consisted of the shortest compound of this series, polysaccharide-aspartic acid [1].

For determination of the quantitative amino acid and carbohydrate composition a sample of glycopeptide, dried to constant weight, was dissolved in a definite volume of water and an exact amount of this solution was hydrolyzed with 20% HCl for 20 hr at 110° for determination of amino acids, and with 1.5 N HCl for 5 hr at 100° for determination of the carbonhydrates. Hexoses were determined colorimetrically, by orcin, from the same solution, without preliminary hydrolysis. Thus was assured maximum accuracy in determination of the ratio of amino acids and carbohydrates in the preparations. Paper separation of amino acids and glucose amine in the hydrolyzates was carried out by successive electrophoresis at pH 2.7 in acetic acid in one direction and chromatography in the system butanol-acetic acid-water in the direction perpendicular to the first. Semiquantitative determination of the amino acids on the chromatogram was carried out by the method of Giri [6].

The N-terminal amino acids in the glycopeptides were determined by the dinitrophenyl method in the reaction with fluorodinitrobenzene at 37° for 8 hr [7].

For detecting the C-terminal amino acids we used the method of hydrazinolysis of the glycopeptides with later dinitrophenyl derivative formation of the free C-terminal amino acids in the variant of Bradbury [8].

RESULTS AND DISCUSSION

From the numerous experiments which we carried out on the separation of glycopeptides it follows that in enzymatic hydrolysis of egg albumin we find not one, but several glycopeptides, and that, depending on the

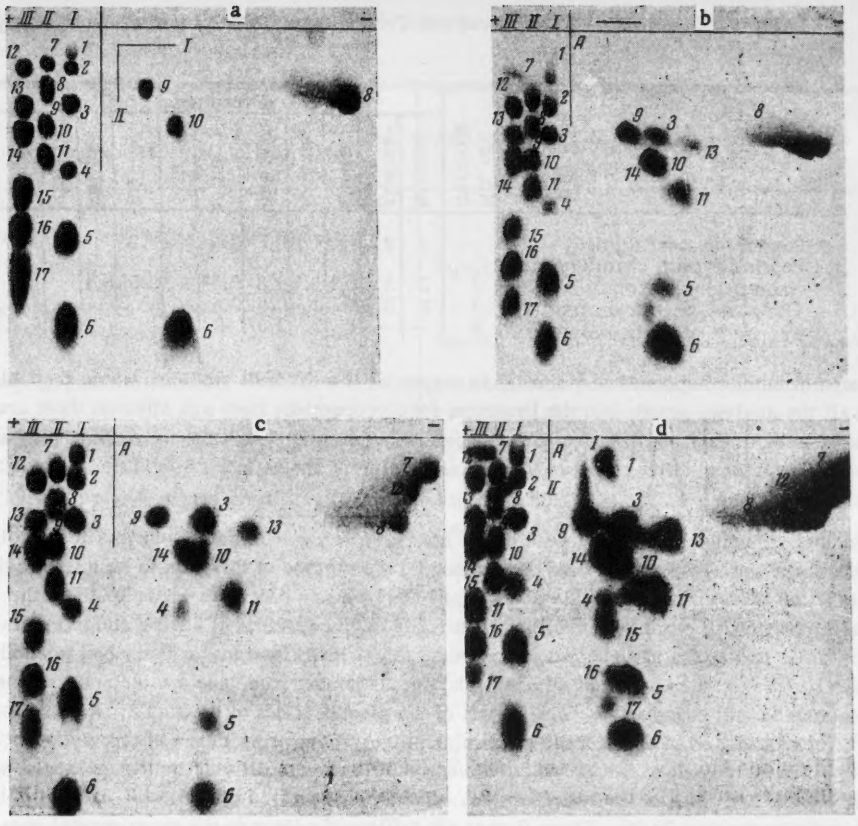

Fig. 2. Electrophoresis-chromatogram of amino acids from glycopeptides. a) Amino acids of fraction 3 of glycopeptides obtained after action of pepsin, trypsin, and chymotrypsin (deep hydrolysis); b) amino acids of fraction 3 of glycopeptides hydrolyzed by the same enzymes, but with incomplete hydrolysis; c) amino acids of fraction 4 of the same hydrolyzate; d) amino acids of glycopeptides obtained after the action of trypsin, I, II, III, mixture of amino acid standards: 1) cystine; 2) histidine; 3) serine; 4) proline; 5) valine; 6) leucine; 7) lysine; 8) glucosamine; 9) aspartic acid; 10) threonine; 11) alanine; 12) arginine; 13) glycine; 14) glutamic acid; 15) tyrosine; 16) methionine; 17) phenylalanine.

enzymatic procedure used, the glycopeptides differ in length of the peptide chain. Differences in amino acid composition are seen from the two phase electrophoretogram-chromatogram (Fig. 2) of the glycopeptides hydrolyzed to amino acids. As the figure shows, the number of amino acids rises with shift from the glycopeptides of deeper enzymatic splitting (Fig. 2a) to glycopeptides of less deep splitting (Fig. 2c and d).

We studied in more detail the largest fraction 3.

In determining the N-terminal peptides in this fraction we always found aspartic acid and among the somewhat less deep products of splitting, traces of tyrosine. In the C-terminal peptide chains, in the glycopeptides of fraction 3 obtained in the purest form, we found not less than two amino acids (threonine and leucine); in the case of less deep hydrolysis we also found serine. Since mixtures of amino acids and peptides were fully excluded by carrying out the purification process, these results show that fraction 3 consists of a mixture of glycopeptides very similar in properties, differing by 1-3 amino acid residues. Hence, even after separation by electrophoresis of the isolated glycopeptide fractions they were not entirely homogeneous. This is also indicated by the fractional number of amino acid residues in the glycopeptides (Table 1).

TABLE 1. Quantitative Amino Acid and Carbohydrate Composition of Some Glycopeptides

Hydrolysis by enzymes	No. of fractions	No. of residues											
		hexoses	glucosamine	aspartic	leucine	threonine	serine	alanine	glycine	valine	glutamic	lysine	tyrosine
Pepsin-pancreatic juice	—	5	3	1,0	1,6	0,5	0,4	0,2	0,3				
Pepsin, trypsin, chymotrypsin, incomplete hydrolysis	3	5	3	1,1	1,7	0,8	0,55	0,3	0,55	0,1			0,16
The same, deep hydrolysis	3	5	3,3	1	2	0,6	0,1						
The same, deep hydrolysis	4	—	—	1	2	0,57	+	+	+	+	+	0,25	+

Full separation of compounds very similar in properties is a difficult problem, which probably explains the fact that in all the analyses reported in the literature for glycopeptides from egg albumin there are fractional amounts of amino acid residues [7, 9 , 10]. It also follows from table 1 that the polysaccharide part of the glycopeptide fraction contains in all cases hexoses and glucosamine in the ratio 5 : 3, and this is obviously the same in all fractions.

The molecular weight of the glycopeptides of fraction 3 (after incomplete enzymatic hydrolysis by pepsin and a mixture of trypsin and chymotrypsin) was determined, by the method of diffusion, to be in the region of 1900,* the hexose content was 36% and the acetylglucosamine, 27.2%. We can calculate what should be the average molecular weight of the glycopeptides with the composition we found of five residues of hexose and three glucosamine residues. The polysaccharide part of the glycopeptides, according to our investigations, as described in another study [11], should contain besides these compounds, another unknown component and have a molecular weight about 1575. The average molecular weight of the whole peptide part of the glycopeptides of fraction 3, from the analysis which we ran, was 517 (for calculation of the average molecular weight of the peptide part of the glycopeptides the observed number of the residues of each of the amino acids was conditionally multiplied by the molecular weight of these residues and totaled). In sum, for the glycopeptides we obtained 2092; in this case the content of hexose should equal 38.7% and glucosamine 29.2%. These figures are sufficiently close to the experimental ones.

Table 2 shows the gradually increasing complexity in composition of the glycopeptides with lengthening of the peptide chain. Since the quantitative data give two residues of leucine per glycopeptide, leucine is repeated twice in the list of amino acids in the sequence which express the amino acid composition of the glycopeptides of different lengths.

In the composition of fraction 4, obtained after hydrolysis by pepsin, trypsin, and chymotrypsin and with basic properties, to judge by its rate of mobility in electrophoresis, we find lysine which is not present in fraction 3. The same amino acid is present in the glycopeptides from the pure trypsin hydrolyzate, but the latter also contains amino acids which are not in fraction 4. Since lysine should occupy the C-terminal position in the trypsin glycopeptide, the other new amino acids in this glycopeptide as compared to fraction 4, namely: tyrosine, methionine, and cystine, should be found in the N-terminal chain and serve to prolong it from the side of aspartic acid. Such a suggestion agrees with the fact that tyrosine was already found in this position in one glycopeptide by Cunningham [9], and also by us in the present work, but with conditions of specificity of action the bond between aspartic acid and tyrosine should not be ruptured by trypsin.

Thus, in the hydrolysis by all the proteolytic enzymes of the digestive tract, pepsin, trypsin, chymotrypsin, and carboxypeptidase, there was liberated the shortest glycopeptide, namely: polysaccharide-aspartic acid; under the action of pepsin-pancreatic juice there are formed short glycopeptides close in composition to those which were found by other authors [7, 9, 10]. Here there is formed a very slight amount of polysaccharide-aspartic acid (shown as a trace in fraction 2) because of the content of carboxypeptidase in pancreatic juice; finally, by replacing pancreatic juice by pure trypsin and chymotrypsin there is a longer glycopeptide chain (fraction 2 is absent, fraction 4 appears). Under the action of trypsin alone there results a glycopeptide or glycopeptides with basic properties and a still longer peptide chain.

* The determination was carried out by Yu. I. Khurgin, to whom we express our thanks.

TABLE 2. Amino Acid Composition of Glycopeptides Isolated from Egg Albumin by the Action of Different Proteases

Enzymes and conditions of hydrolysis	Amino acids of peptides and their probable sequence
Pepsin, trypsin, chymotrypsin, carboxypeptidase	Aspartic
Pepsin, trypsin, chymotrypsin (deep hydrolysis) fraction 3	Aspartic, leucine, threonine, leucine (serine)*
Pepsin, pancreatic juice	Aspartic, leucine, threonine, leucine, serine (alanine, glycine, glutamic)
Pepsin, trypsin, chymotrypsin, (incomplete hydrolysis), fraction 3	Aspartic, leucine, threonine, leucine, serine, alanine, glycine, (glutamic, valine)
The same enzymes and conditions, fraction 4	Aspartic, leucine, threonine, leucine, serine, alanine, glycine, (glutamic, valine, proline, lysine)
Trypsin	Aspartic, leucine, threonine, leucine, serine, alanine, glycine, glutamic, valine, proline, lysine, tyrosine cystine, methionine, tyrosine, cystine, methionine.

*The parentheses include amino acids present in small amounts.

SUMMARY

It is shown that the carbohydrate-protein bond in egg albumin is not split by the action of proteolytic enzymes (pepsin, trypsin, chymotrypsin, pancreatic juice).

Under the action of different proteases or their combinations glycopeptides are formed in the hydrolyzate from egg albumin; they have the same polysaccharide portion, but differ in length and composition of peptide chain.

By comparing the composition of the different glycopeptides we find the approximate order of arrangement of amino acids in the polypeptide chain of egg albumin directly bound to the polysaccharide.

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THE VALUE OF THE FREE ENERGY OF THE HYDROLYSIS REACTION OF PHOSPHOSERINE

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The high rate of incorporation of radioactive phosphorus in the structure of phosphoproteins of various tissues [1-4] apparently indicates that these phosphoproteins play a special role in the metabolism of phosphate groups. During studies on the renovation rate of the phosphoproteins of brain tissue, it was found that almost all the phosphate included in these proteins is attached to serine, and that it can be isolated as phosphoserine [5, 6]. In relation to these facts, the determination of the value of the free energy of the hydrolysis reaction of phosphoserine constitutes the basis for the elucidation of the direction of the reaction during phosphorus exchange between phosphoproteins and other substances. No data have been yet published on the value of the free energy of the hydrolysis reaction of phosphoserine, except an indication by Rabinowitz and Lipmann[7] to the effect that for phosphoproteins, the energy of the reaction of the hydrolytic fission of a phosphate group is by 2.9 kcal lower than the value of the free energy of the hydrolysis reaction of ATP.

In the present work, we attempted to determine the value of the free energy of the hydrolysis reaction of phosphoserine from the equilibrium constant in the presence of kidney phosphatase:

$$\text{Serine} + \text{phosphate} \underset{Mg^{++}}{\overset{\text{phosphatase}}{\rightleftharpoons}} \text{phosphoserine} + H_2O$$

METHODS

Kidney phosphatase was prepared from pig [8] and rat [9] kidneys. The activity of phosphatase was checked before each experiment by its action on phosphoserine. Phosphoserine which was used as a carrier was synthesized by a chemical procedure according to Plimmer [10] from serine and crystalline phosphoric acid in the presence of P_2O_5. The phosphoserine thus prepared was chromatographically identical with the phosphoserine isolated from casein after an acid hydrolysis of the latter [11]. The experiments were carried out with a reaction mixture consisting of a serine solution in a 0.9 M medinal-veronal buffer of the required pH value, $NaH_2P^{32}O_4$, and kidney phosphatase. Magnesium chloride (0.05 mg/ml) was added to the samples as an activator of phosphatase. The samples were incubated at 35° for 1-4 hr. The reaction was stopped by the addition of trichloroacetic acid (50 mg/ml).

Simultaneously with each experiment, a blank test was carried out, which differed only by containing a phosphatase inactivated by boiling. Investigation of the blank experiment allowed in each case to determine to what extend the subsequent purification of the samples was sufficient for the complete removal of contamination with radioactive P_{inorg}.

During the reaction, a certain amount of phosphoserine was synthesized from serine and phosphate; the amount of this phosphoserine could be determined only with the help of the radioactive labelling. In order to isolate the phosphoserine synthesized in the experimental medium, the samples were treated with a definite amount of unlabelled phosphoserine, prepared by a chemical method, to act as carrier. For carrying out the determination of the amount of synthesized phosphoserine, it was necessary to purify the preparation from labelled P_{inorg}. The main amount of P_{inorg} was precipitated by Delory's method [12]. The further purification of phosphoserine from radioactive P_{inorg} was attained by its repeated (3-11 times) precipitation with ethanol. Then, the precipitate of phosphoserine was dissolved in a small volume of water, and for its further purification from contaminating P^{32}, the solution of phosphoserine was chromatographed on paper. Two or three parallel chromatograms were run with both the

TABLE 1. Equilibrium Constant and Value of the Free Energy of the Hydrolysis Reaction of Phosphoserine in a Medium at pH 9.7

No. of expt.	Concentration of the substances participating in the reaction: Serine, M	Phosphate M	Radioactivity of phosphate, imp/min	Duration of the expt. in min	Amt. of phosphoserine carrier mg P/sample	No. of chromatograms	Amt. of synthesized phosphoserine (10^{-8} M)	K	ΔF_0, kcal
1	0,0093	0,0350	$10,95 \cdot 10^8$	60	3,870	1	1,50	217	−3,29
						2	0,74	437	−3,72
						3	0,94	341	−3,57
2	0,0094	0,0270	$3,32 \cdot 10^8$	90	3,870	1	0,44	577	−3,89
						2	1,51	168	−3,14
3	0,0079	0,0448	$5,64 \cdot 10^8$	105	1,172	1	2,08	185	−3,20
						2	1,91	201	−3,25
4	0,0099	0,0150	$4,65 \cdot 10^8$	200	0,280	1	0,86	180	−3,18
						2	0,82	180	−3,18
5	0,0090	0,0202	$1,93 \cdot 10^8$	120	0,633	1	0,53	340	−3,57
						2	0,55	327	−3,55
	Mean							287	−3,41

experimental and the blank solutions. Each chromatogram comprised two spots: either control solution, or experimental solution, and phosphoserine prepared by a chemical procedure, as comparison sample.

The following mixture was used as a solvent: n-butanol–ethanol–0.005 N HCl (3 : 2 : 2). The solvent was allowed to flow in the descending direction for 48 hr. During that time, phosphoserine travelled 12-15 cm, and P_{inorg} – 40-50 cm. The main part of P^{32} was thus separated from phosphoserine on the chromatogram. The location of phosphoserine in experiments with the experimental or control samples was determined by comparison with the comparison sample of phosphoserine which was revealed with ninhydrin or the molybdenum reagent of Haines and Isherwood. As both of these revealing agents destroy phosphoserine, direct detection of phosphoserine in experimental or control samples with the help of these reagents has not been carried out.

The phosphoserine spot was cut out at the level indicated by the phosphoserine comparison sample, and extracted with 1 N HCl. The remaining traces of P^{32} in the samples were isolated in the form of phosphomolybdic complex, with isobutanol, by the method of Berenblum and Chain [13]. Extraction was repeated two or three times. The last isobutanol extract was free of P^{32}. Then, the samples were incinerated. Their P_{inorg} content was determined by Fiske and Subbarow's method, and their radioactivity was assayed on a B-2 assembly fitted with a AS-2 counter. The equilibrium constant was calculated with the help of the formula:

$$K = \frac{(\text{serine})\,(\text{phosphate})}{(\text{phosphoserine})}$$

The free energy of hydrolysis (ΔF_0) of phosphoserine was calculated with the help of the formula:

$$\Delta F_0 = -RT\log_e K,$$

where R is the gas constant ($0.00198 \frac{\text{kcal}}{\text{m.deg}}$), and T is the absolute temperature (273 + 35°).

RESULTS AND DISCUSSION

In order to calculate the equilibrium constants of the reaction it was necessary to measure the concentration of the substances involved under equilibrium conditions of the system. Control experiments on the hydrolysis of phosphoserine with kidney phosphatase showed that hydrolysis is practically completed during 30 min of incubation. In our experiments, we deliberately chose a longer time (from 60 to 240 min) for the phosphatase reaction in order to ensure the attainment of the equilibrium state.

As one can see from the given data (expts. 1-5, Table 1), the duration of the experiment did not influence the value of the equilibrium constant, thus proving that the equilibrium state was really attained.

TABLE 2. Equilibrium Constant and Value of the Free Energy of the Hydrolysis Reaction of Phosphoserine in a Medium at pH 7.0-7.1

No. of expt.	Concentration of the substances participating in the reaction		Radioactivity of phosphate, imp/min	Duration of the experiment, in min	Amt. of phosphoserine carrier (mg P/sample)	No. of chromatograms	Amt. of synthesized phosphoserine (10^{-6} M)	K	ΔF_0, kcal
	serine, M	phosphate, M							
6	0.0092	0.0308	$7.10 \cdot 10^8$	210	0.753	1	0.26	1083	− 4.28
						2	0.55	522	− 3.85
						3	0.27	928	− 4.18
7	0.008	0.0260	$5.80 \cdot 10^8$	240	0.381	1	0.70	273	− 3.44
						2	0.37	560	− 3.92
	Mean							673	− 3.95

Considerable deviations from the mean value of the equilibrium constant (up to 100%) were observed in separate experiments. Apparently, this is due to the negligible amounts of phosphoserine which had to be estimated (thus, in expt. 7, only 5×10^{-9} g of labelled phosphoserine was determined in 10 μg of P isolated from the phosphoserine carrier). In the determination of ΔF values, such extremes in the measured amounts lead to differences which are not greater than 0.3 kcal/mole.

The equilibrium state of the hydrolysis and synthesis of phosphoric esters is under the influence of the pH of the medium. This is due to a change in the dissociation of acid groups. The first OH group of free phosphoric acid or of its derivatives, is fully ionized, whereas the third OH group ($K = 4.5 \times 10^{-12}$) of phosphate practically does not dissociate at pH 9.7 or lower, and does not influence the state of the medium. The acid properties of free phosphoric acid and those of its esters in a pH region close to the neutral are determined exclusively by differences in the dissociation of the second OH group. The combination of phosphate with creatine and glycerine strongly influences the dissociation of the second OH group, the dissociation constant of this group being increased (thus, for free phosphoric acid it equals 1.5×10^{-7}, and for glycerophosphate, 4.7×10^{-7}).

Hydrolysis of phosphoric acid esters is accompanied by bonding of hydrogen ions, and their synthesis, by liberation of these ions:

$$\text{serine} + \text{phosphate} \rightleftharpoons \text{phosphoserine} + \text{water} + n\text{H}^+,$$

where $\underline{n}$ is a simple fraction.

In agreement with Le Chatelier principle, a pH change in the direction of increased acidity displaces the equilibrium state in the direction of decreased phosphoserine concentration. The change of the equilibrium constant of the hydrolysis reaction of phosphoric acid esters due to a change in the hydrogen ion concentration in the range close to physiological concentration can be calculated with the help of Ginodman's formula [14]:

$$\frac{K_{ph} + H_1^+}{K_{sph} + H_1^+} \cdot \frac{K_{sph} + H_2^+}{K_{ph} + H_2^+} = \frac{K_1}{K_2},$$

where H_1 and H_2 are the hydrogen ion concentrations for alkaline and acid medium, respectively; K_1 and K_2, the equilibrium constants for alkaline and acid medium, resp.; K_{ph} and K_{sph}, the dissociation constants of the second acid group in free phosphoric acid and phosphoserine, resp. According to Österberg [15], $K_{sph} = 10^{-5.67}$. Calculation with the help of this formula for a change from pH 9.7 to pH 7.0 shows that the equilibrium constant is changed by a factor of 1.6:

$$\frac{10^{-6.82} + 10^{-9.7}}{10^{-5.67} + 10^{-9.7}} \cdot \frac{10^{-5.67} + 10^{-7}}{10^{-6.82} + 10^{-7}} = \frac{1}{1,6}.$$

Thus, if we have the constant equal to 287 for pH 9.7, then, for pH 7.0 we must expect it to be equal to 459. The

TABLE 3. Value of the Free Energy of Hydrolysis of Some Phosphoric Acid Esters

No.	Compound	pH	kcal	Author
1	α-glycerophosphate	8.5	2.20	Mayerhof and Green [16]
2	3-Phosphoglyceric acid	8.5	3.00	Mayerhof and Oesper [17]
3	Glucoso-6-phosphate	8.5	3.00	Mayerhof and Green [16]
4	" "		2.45	Ginodman [14]
5	Fructoso-6-phosphate	8.5	3.00	Mayerhof and Oesper [16]
6	ATP	7.0	7.6	Robbins and Boyer [18]
7	"	7.25	5.6-6.2 *	Vladimirov et al. [19]
8	" ω	7.0	6.8	Atkinson et al.[20]
9	Phosphoserine	9.7	3.41	Data from the present work.
10	"	7.0	3.95	" " " " "

* If one takes the ΔF_0 value of the hydrolysis of glucoso-6-phosphate as equal to -2.45 [4], then the calculated value for the hydrolysis of ATP is $\Delta F_0 = 5.6$ kcal; if one uses Mayerhof and Green's value, i.e., 3.00 [3], then the ΔF_0 value for ATP becomes 6.2 kcal.

experimental results (Table 2) showed that the equilibrium constants are really greater in neutral medium than in alkaline medium. Although the experimental difference between the equilibrium constants at pH 7.0 and pH 9.7 is not in full agreement with the calculated difference, nevertheless, it is not much different from it.

After one has determined the equilibrium constant of the reaction, one can calculate the ΔF value of phosphoserine hydrolysis. Under standard conditions, when the concentration of the substances was 1 M, at pH 9.7, this ΔF value was found to be -3.41 kcal and at pH 7.0-7.1-3.95 kcal.

By comparing the ΔF_0 value thus obtained with the literature data for some phosphoric acid esters (Table 3), one can conclude that phosphoserine is a compound possessing a higher bond energy than the other esters of phosphoric acid with various alcohols.

As the introduction of phosphoserine in the composition of a protein apparently does not substantially influence the ΔF_0 value of the hydrolysis of a phosphoserine bond, then phosphoproteins must also be considered as substances having a somewhat higher ΔF_0 of hydrolysis than a whole series of phosphorus compounds constituting the intermediate glycolysis products. It is noteworthy that Sidbury and Najjar [21] found that the ΔF_0 value of phosphoglucomutase is 3.9 kcal.

Such a ΔF_0 value for the phosphoproteins ensures the thermodynamic reversibility of the exchange of phosphate groups between phosphoproteins and phosphoric esters. The above mentioned facts are in good agreement with the assumptions that phosphoproteins are catalytically active proteins which participate in metabolism [22]. It has already been proved that some enzymes catalyzing the transfer of phosphate groups, viz., hexokinase [23], phosphoglucomutase [24-26], and phosphorylase [27, 28] are phosphoproteins in which phosphoric acid is combined as phosphoserine.

The data obtained allow us to continue the discussion of the ΔF_0 value of the hydrolysis of ATP. In the latest investigations, this value varies within the range 5.6-7.6 kcal (Table 3, [6-8]). If the hydrolysis energy of phosphoproteins is the same as in the case of phosphoserine, and if we accept Rabinowitz and Lipmann's data [7] according to which the transfer of a phosphate group from ATP to phosphoproteins is accompanied by a free energy change of 2.9 kcal, then the free energy of hydrolysis of ATP would be 2.9 kcal larger than the hydrolysis energy of phosphoserine, i.e., it would lie between 6.3 kcal (pH 9.7) and 6.85 kcal (pH 7.0). The calculated value of ΔF_0 for ATP is thus very close to the experimental values, especially to those of Atkinson et al.

SUMMARY

The free energy of the hydrolysis reaction of phosphoserine, as determined according to the equilibrium constant of the phosphatase reaction, has a mean value of 3.95 kcal under standard conditions.

The free energy of hydrolysis of ATP is slightly lower than 7 kcal per mole of phosphoric acid.

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EFFECT OF SARCOLYSIN PEPTIDES ON THE METABOLISM OF PURINE NUCLEOTIDES IN TUMOR CELLS

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In the search for new antitumor agents, combining in themselves properties of haloid alkylamines and antimetabolites, compounds have been synthesized in which the chloroethylamine part of the molecule was combined with substances produced by nature, specifically by amino acids and peptides. Knunyants and others [1] synthesized sarcolysin peptides and their esters with the following general formula:

where R is an amino acid residue (methionine, valine, phenyl alanine, etc). Larionov and Sof'ina [2, 3], investigating the antitumor activity of these preparations, showed that their action was highly selective for some tumors, depending on the nature of the amino acid. In this connection it was interesting to investigate the mechanism of action of sarcolysin peptides, since the data obtained could furnish many possibilities for the creation of new antitumor agents.

In the first investigation undertaken in this direction [4] we studied the process of binding and the cleavage of sarcolysin peptides (sarcolysyl-valine ethyl ester and sarcolysyl-methionine ethyl ester) in ascites cells of animal tumors (in vitro) as well as their influence on the synthesis of proteins and nucleic acids in the same tumors. This work showed that a considerable portion of the sarcolysin peptides were combined with cellular proteins and polypeptides in the incubation process. It was shown at the same time that there was a decrease in DNA and protein synthesis in the cells and an accumulation of some tumor substances in the non-cellular incubating medium which absorbed UV light. Determination of the nature of this substance as one of the possible means of approaching an understanding of the mechanism of action of the agents studied, was the object of the present investigation.

Accumulation of substances absorbing UV light in the incubation medium under the influence of alkylating compounds was observed earlier in experiments with microorganisms [5]. There have also been a few indications in the literature of the accumulation of acid soluble substances absorbing UV light in microorganisms and in mammalian organs under the influence of ionizing radiation [6-10].

METHODS

The work was carried out on the following animal ascitic tumors: sarcoma 180, Ehrlich carcinoma and sarcoma 37 of mice, as well as ovarian cancer and Zazhdel' rat hepatoma. Cells washed from ascitic fluid were incubated in a Warburg apparatus in Krebs-Ringer-phosphate buffer in the presence of sarcolysin peptides (sarcolysyl-valine ethyl ester and sarcolysyl-methionine ethyl ester). The preparations were usually added in a dose of 250 μg per ml incubation medium. For comparison, experiments were set up with sarcolysin and embichine in equimolecular quantities with respect to the sarcolysin peptides. The incubation was carried out under aerobic conditions for 30 min, 1 and 2 hours. At the end of the experiment the cells were removed from the incubation medium by centrifugation (10 min at 2,000 rev/min) and the supernatant liquid was subjected to paper chromatography in the solvent, isopropanol-5 N HCl-ethanol (6 : 2 : 2) (solvent 1). The chromatograms were examined in the ultrachemiscope. The spots absorbing UV light were further eluted and investigated by means of chromatography and spectrophotometry as well as chemically. Usually 10-15 such spots were cut out and eluted in 0.2 N HCl in the cold for a day. The eluates were investigated in the spectrophotometer at ranges of 230-310 mμ (Millimicrons) at different pH's (in 0.2 N HCl at pH 7.0 and 10.0). The eluate obtained was then dried in a vacuum to a volume of 0.5-1 ml

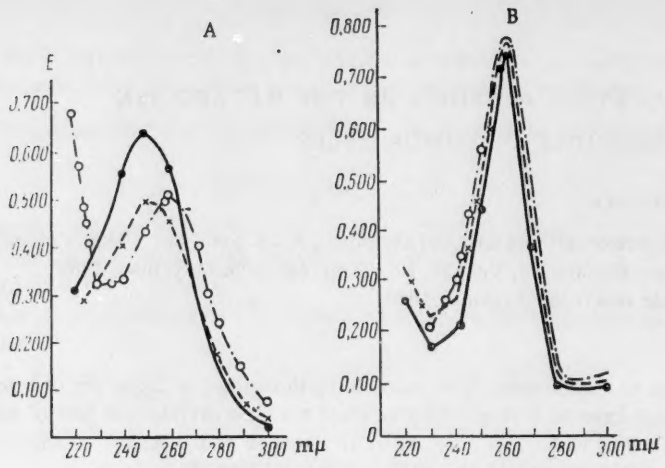


Fig. 1. Spectrum sarcolysyl-valine ethyl ester (B) 20 μg/ml and eluate of spot III (A). Incubation with sarcolysyl-valine ethyl ester: A) 1 hr; B) 3 hr. Continuous lines, pH 2.0; dotted with crosses, pH 7.0; with circles, pH 10.0.

and subjected a second time to paper chromatography in isopropanol (70%, saturated with water) in an atmosphere of ammonia (solvent 2). The chromatograms were dried for a day in air and again examined in the ultrachemiscope. The spots found were also eluted with 0.2 N HCl. Part of the eluates were investigated in the spectrophotometer; another portion was investigated chemically for phosphorus and ribose content after evaporation in a vacuum.

Ribose was determined by means of Bial's reagent. Phosphorus was determined after combustion in 10 N H_2SO_4 with $SnCl_2$, and the optical density, in the electrophotocolorimeter, was determined.

The tumor cells removed from the incubating medium by centrifugation were treated in the cold with $HClO_4$. The $HClO_4$ extracts obtained were neutralized with KOH, dried in a vacuum and investigated by means of one-dimensional paper chromatography in solvent 1. The chromatogram was dried and examined in the ultrachemiscope. The spots noted on the chromatogram were eluted with 0.2 N HCl and investigated by the same methods used for the spots absorbing UV light. Part of the eluates were hydrolyzed in 1 N HCl at 100° for 20-30 min. The hydrolyzates were evaporated on a water bath to remove HCl; the dry residue was dissolved in a minimum quantity of water and deposited on a chromatogram. The free purine bases obtained by hydrolysis were identified by paper chromatography in solvents 1 and 2. As verification adenine, hypoxanthine, xanthine and guanine were deposited on the same chromatogram.

RESULTS OF INVESTIGATION

Investigation of the Cell-Free Incubation Medium

On incubation of ascitic tumor cells with sarcolysin peptides, we observed spots with R_f 0.14-0.15, intensively absorbing UV light, on the chromatogram of the cell-free incubation medium in an experiment with sarcoma 180, Ehrlich carcinoma and ovarian cancer (only with methionine-sarcolysin) (Fig. 1, spot III). In experiments with sarcoma 37 and Zazhdel' hepatoma, as well as in the controls, the formation of similar spots was not observed. Neither were there any UV light-absorbing substances formed when these tumors were incubated with embichine and sarcolysin.

Thus these sarcolysin peptides, as distinguished from embichine and sarcolysin, have a selective action with respect to some animal ascitic tumors, and cause the formation of substances absorbing UV light in the incubation medium.

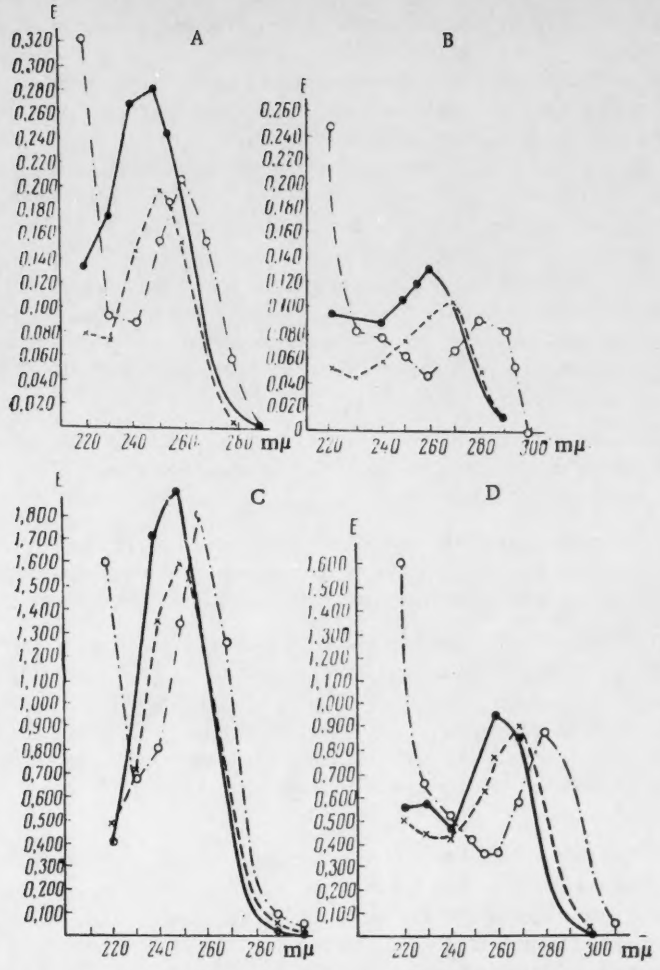


Fig. 2. Spectrum of the eluate of spot IIIa (A), IIIb (B), hypoxanthine (C) and xanthine (D). Incubation 3 hr with sarcolysyl-valine ethyl ester. Solid line, 0.2 N HCl; dotted line with crosses, pH 7.0; with circles pH 10.0.

According to data in the literature, it can be assumed that this substance is formed and accumulated in the incubation medium because of the inhibition of nucleic acid synthesis, and consequently it could be a nucleic acid precursor.

Thus, Davidson et al. [6] investigating the effect of embikhin on the incorporation of labelled precursors of nucleic acids (formate-C^{14}, adenine-8-C^{14}, $P^{32}O_4$ and others) in Ehrlich ascites mouse carcinoma MC-1M and adenocarcinoma 755 showed that while the synthesis of nucleic acids is decreased, the synthesis of acid-soluble adenine nucleotides, on the contrary, is increased 3-4 times. Kanasir and Errera [7] reported on the accumulation of deoxyribonucleotides in the medium 30-40 min after UV irradiation of E. coli. These substances the authors identified as precursors of DNA, accumulating as a consequence of a blocking of its synthesis. Billen [8] identified the substance absorbing UV light from the supernatant liquid obtained from metabolizing cellular suspensions of E. coli after roentgen irradiation. Ten per cent of the total absorption was thereby shown to be due to ATP and 60% to free base, ribosides or mononucleotides. Adenine, guanine, uracil, and cystosine were present either as free bases or in the form of ribosides or ribonucleotides.

Loofbourow et al. [9] irradiating yeast cells with UV-rays, observed conversion of nucleotides and nucleosides, composed of adenine (85%) and guanine derivatives in the suspending medium. The author considered that changes in the synthetic process took place under the influence of the damaged agents, resulting in the accumulation of the substances in the cells and their passage into the medium. Accumulation of deoxyribotides in rat thymus 1 hr after roentgen irradiation was observed by Ord and Stocken [10]. They showed that mono-, di- and triphosphorylated deoxyribonucleotides, accumulated in $HClO_4$ extracts; nucleotide bases were cytosine, uracil, adenine and guanine. These observations were soon confirmed [11, 12] by statements that inhibition of DNA synthesis after irradiation is explained not by the absence of its precursors, but inhibition of its polymerization. This idea was confirmed by the statements of Davidson et al. [6], that the biosynthesis of nucleic acids could be regarded as a three-step process, in which the second and third steps, that is the formation of di- and triphosphates, as well as the condensation of triphosphate is inhibited by chloroethylamines. The proposal that the accumulation of substances absorbing UV-light is a consequence of a decreased DNA synthesis was verified by our observations on the inhibition of DNA synthesis in the presence of sarcolysyl-valine [4]. In addition to this, it could be considered probable that the UV-light absorbing substance, was a decomposition product of nucleic acids existing in the cell.

In order to determine the nature of the substance formed in the cells under the influence of sarcolysin peptides, and also to explain the possible mechanism of its formation, we undertook a study of eluates of spot III and acid soluble products of cell nucleic acids by paper chromatography and spectrophotometry.

Spectrophotometric investigation of eluates obtained from spot III showed that the spot was not a sarcolysin peptide, since its spectrum was different (Fig. 1, A). Since the spectra of the sarcolysin peptides (sarcolysyl-methionine and sarcolysyl-valine) are similar, we used the spectrum of sarcolysyl-valine for comparison (Fig. 1, B).

For more precise identification, the eluate from spot III was again subjected to paper chromatography on solvent 2. Two more spots absorbing UV-light were thereby discovered on the chromatogram; the more intense had an $R_f = 0.45$ (IIIa) and the less intense an $R_f = 0.30$ (IIIb), (Fig. 2, A and B). The eluates of these spots did not contain either phosphorus or ribose. The spectrum characteristic for spot IIIa had a maximum absorption of light at 250 mμ in acid and neutral media which was displaced towards 260 mμ in alkaline pH. The spectrum of spot IIIb differed in that its maximum absorption in acid medium was between 230 mμ and 260 mμ, in neutral medium at 270 mμ, and on alkalization shifted to 280 mμ.

Investigation of the pure control chromatograms of hypoxanthine and xanthine in the same solvents showed that the R_f of hypoxanthine was equal to 0.45 and of xanthine, 0.30. The eluates of these spots in 0.2 N HCl gave spectra corresponding to hypoxanthine with the spectrum of spot IIIa, and to xanthine, with the spectrum of spot IIIb (Fig. 2, C). In this manner, these data demonstrate that spot IIIa was identical in its chromatographic and spectrophotometric properties with hypoxanthine, and spot IIIb with xanthine.

In other words, the substance which accumulates in the incubation medium and absorbs UV light is not a single compound, but is a mixture of two compounds, hypoxanthine and xanthine.

Investigation of Acid Soluble Cellular Extracts

When $HClO_4$ extracts of cells were chromatographed in solvent 1, two distinct spots were observed on the chromatogram which absorbed UV light and had R_f's of 0.24-0.26 (spot 1) and 0.18 (spot 2). Another spot with an R_f of 0.13 (spot 3) could be seen in $HClO_4$ extracts of sarcoma 180.

Spot 1, according to its R_f (0.24-0.26) could coincide with adenylic acid (R_f 0.27-0.28). Its spectrum (Fig. 3) had an absorption maximum at 260 mμ and suggested the spectrum of adenine and its nucleotides.

Chromatography of an hydrolyzate of spot I resulted in a spot which according to its R coincided with free adenine (R 0.17). The eluate of spot 1 gave a positive reaction for ribose and phosphorus. Thus, the spot with R 0.24-0.25 coincided with adenine nucleotide and, most probably, to adeninemonophosphate.

Spot 2 according to its R_f (0.18) could have coincided with guanilic acid (R_f 0.18), inosinic acid (R_f 0.15) or adenosine (R 0.16). However, the final nature of this compound had not been established. The spot contained ribose and phosphorus, and on acid hydrolysis of the eluate, chromatography of the hydrolyzate showed the presence of hypoxanthine. Thus it could be supposed that this spot corresponded to inosinic acid.

Spot 3 on hydrolysis also gave hypoxanthine; it is possible that it was inosinedi- or triphosphate.

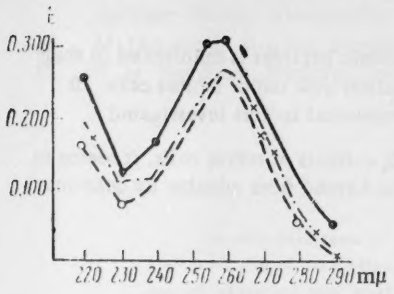


Fig. 3. Spectrum of the eluate of spot 1 from the chromatogram of the chloric acid cellular extract. Designations the same as in Fig. 1.

The spectrophotometric investigation of the chromatograms revealed that the concentration of substances found in acid soluble cell fractions did not remain constant during incubation. The character of these changes altered considerably with the addition of sarcolysin peptides to the incubation medium.

It is evident from the curves presented in Fig. 4 that the most pronounced changes in the experimental samples compared with the controls occurred in the adenine nucleotide content (Fig. 4 A). It decreased somewhat in the control during the first hour of incubation; this decrease, however, was suspended on further incubation. In the presence of sarcolysin peptides, unlike the controls, a sharp decrease in adenine nucleotides was observed in the interval between 1 hr and 2.5 hr incubation. It

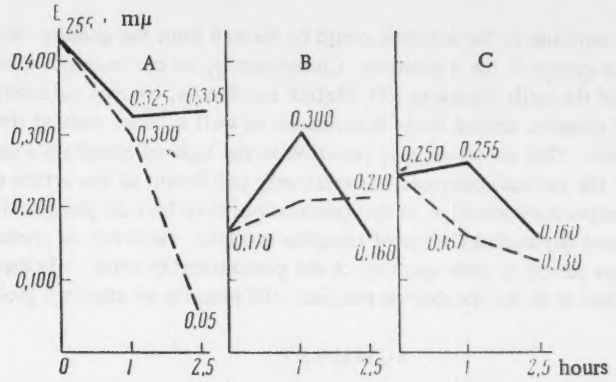


Fig. 4. Change in the amount of acid soluble cellular components in the process of their incubation with sarcolysin peptides. Dotted line, incubation with sarcolysin peptides; solid line, control. A) spot 1 (R 0.24); B) spot 2 (R_f 0.18); C) spot 3 (R_f 0.10).

must be noted that the amount of lost adenine nucleotide corresponded approximately to the quantity of hypoxanthine accumulated during this same period in the incubation medium.

The resulting curve (Fig. 4, B) shows that an intensive synthesis and cellular accumulation of inosinic acid apparently occurred in normal cells at the beginning of incubation. Further, it was apparently used for the formation of adenine and guanine nucleotides and thus its concentration decreases. In the presence of sarcolysin peptides an insignificant increase in the concentration of inosinic acid occurred during the experiment. However, the inosinic acid formed in this case, apparently was not used for the formation of adenine and guanine nucleotides and thus its concentration during long incubation did not decrease. The accumulation of inosinic acid was either the result of its synthesis or the result of the deamination of adenylic acid with subsequent formation of hypoxanthine. The solution of these problems, however, is possible only by using labelled precursors.

The content of what is assumed to be inosinediphosphate (Fig. 4, B) is decreased in the presence of the drug in comparison with the controls during the course of the whole incubation.

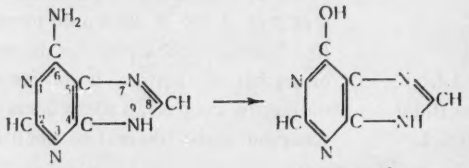
DISCUSSION OF THE RESULTS

Investigating the action of sarcolysin peptides (sarcolysyl-valine and sarcolysyl-methionine) on animal tumor ascites cells, we noted that along with chloroethylamine activity, sarcolysin peptides have a unique action on these cells, which distinguishes them from embichine and sarcolysin. As typical chloroethylamines, they inhibited the

synthesis of nucleic acids, but unlike embichine and sarcolysin, greatly altered the permeability of the cells, as evidenced by the passage of amino acids and peptides into the medium [4].

In the experimental material presented above the specific action of sarcolysin peptides is manifested in their ability to bring about the formation of hypoxanthine and xanthine during incubation with tumor ascites cells. It may be noted that the action of peptides was selective with respect to the experimental tumors investigated.

Comparing the data of the investigation in incubation medium and $HClO_4$ extracts of tumor cells, it seems to us possible to assume that the hypoxanthine found in the incubation medium was formed from adenine by deamination of its amino group in the 6 position:



Adenine (6-aminopurine) → Hypoxanthine (6-oxypurine)

In a similar manner the xanthine in the medium could be formed from the guanine derivatives by means of the deamination of their amino groups in the 2 position. Consequently, we can consider that sarcolysin peptides, acting on the living structure of the cells (sarcoma 180, Ehrlich carcinoma, ovarian carcinoma) were capable of releasing a number of enzyme systems, among them deaminases, as well as those causing the dephosphorylation of acid soluble cellular nucleotides. This seems entirely probable in the light of Needham's data [13] demonstrating that although the basic part of the cellular enzymes was relatively indifferent to the action of haloidalkylamines, there are a group of enzymes especially sensitive to this preparation; they include phosphokinases and enzymes necessary for phosphorylation and dephosphorylation of adenylic residues. However, at present we still cannot say whether this deamination occurs before or after splitting of the purineriboside bond. Whether the deamination process occurs in the cells themselves or in the incubation medium still remains an unsolved problem.

SUMMARY

Sarcolysin peptides, unlike embichine and sarcolysin, when incubated with ascites cells of some tumors (sarcoma 180, Ehrlich carcinoma, ovarian carcinoma) bring about an accumulation in the incubation medium of substances absorbing ultraviolet light.

Chromatographic and spectrophotometric methods identified this substance as a mixture of hypoxanthine and xanthine. Incubation of the cells with the drug simultaneously caused a distinct decrease in adenylic acid, with a concomitant accumulation of hypoxanthine. These data are evidence of a distubance of adenine nucleotide metabolism, manifested in an intensification of their deamination and dephosphorylation.

In conclusion, we would like to extend thanks to A. K Belousova for direction of the work and Z. V. Kudryasheva for technical assistance.

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STRUCTURE OF MANNAN OF DIPHTHERIA BACTERIA

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The application of the periodate oxidation method with the simultaneous determination of the formic acid produced and of the periodate consumed allows to assay the number of 1,6-bonds in a polysaccharide molecule, as well as the number of other bonds, such as 1,2- and 1,4-. An investigation of the products formed as a result of periodate oxidation allows to elucidate the question of the eventual presence of 1,3- bonds in the molecule of the given polysaccharide.

In our previous works [1], we oxidized mannan from diphtheria bacteria with a saturated solution of potassium periodate, and we determined the amount of formic acid produced. On the basis of these experiments, it is now possible to establish that the mannan of diphtheria bacteria is a strongly branched polysaccharide.

In the present communication we present the data obtained during a study of the oxidation of the mannan of diphtheria bacteria with sodium periodate.

EXPERIMENTAL PART AND DISCUSSION

The amount of formic acid produced during the periodate oxidation of mannan was estimated by titration with 0.01 N NaOH using phenolphthalein indicator. The amount of periodate consumed was determined by Fleury and Lange's method [2].

For the elucidation of the eventual presence of 1,3-bonds, mannan oxidized with periodate was submitted to hydrolysis, and the hydrolyzate was investigated by the method of paper chromatography. The hydrolyzate of mannan previously oxidized with periodate could contain intact mannose molecule only on the condition that the mannan molecule contained 1,3-bonds.

The $NaIO_4$ solution used in our experiments was prepared by Rutberg's method [3], viz., a boiling saturated KIO_4 solution was passed through a column filled with the ion-exchange resin KU-2 in its sodium form. The concentration of the $NaIO_4$ solution thus obtained, varied within the limits of 0.18 M and 0.24 M.

In order to select the optimum oxidation conditions, we carried out a number of preliminary experiments in which we changed the periodate concentration and the temperature. Comparison of the results obtained showed that, independently of these factors, the amount of periodate consumed rapidly reached its maximum value which was rather constant, whatever conditions were used. On the other hand, the amount of formic acid produced was increasing gradually, and depended on the concentration of the $NaIO_4$ solution used in the oxidation (within the limits of the concentrations used). The amount of formic acid produced did not reach a constant level during the oxidation time which was equal to 240 hr.

In order to diminish the influence of periodate concentration, we tried to carry out the oxidation in the presence of an acetate buffer at pH 3.6. It was found that the presence of this buffer exerts only little influence on the amount of periodate consumed (Table). However, in its presence, the periodate concentration does not exert any observable influence on the amount of formic acid produced. Another advantage of oxidation in the presence of an acetate buffer is the comparatively rapid attainment of the constant level of formic acid produced and periodate consumed. Therefore, for the determination of the number of the different bonds in the mannan molecule, we decided to carry out periodate oxidations in the presence of an acetate buffer. The accuracy of our method was preliminarily controlled by using a dextran preparation, the so-called "intradex".*

* This preparation has been kindly supplied to us by E. L. Rosenfel'd of the Institute of Biological and Medical Chemistry, Academy of Medical Sciences of the USSR, Moscow.

Production of Formic Acid and Periodate Consumption Under Different Experimental Conditions (Calculated in moles w.r. to the glucose residue. Oxidation was carried out for 72 hr.)

Experimental conditions	Formic acid produced		$NaIO_4$ consumed
	Titration with 0.01 N NaOH under air	Titration with 0.01 N $Ba(OH)_2$ under nitrogen	
Oxidation in the absence of buffer	0.92	0.93	1.92
Oxidation in the presence of buffer	1.07	–	2.08
Literature data [4]	0.92	–	1.90

We were also interested to compare the results obtained with titration of the formic acid produced with 0.01 N NaOH under ordinary conditions and with 0.01 N $Ba(OH)_2$ in an atmosphere of nitrogen. The data obtained showed that titration of the formic acid formed by different techniques gives practically the same results, and that operating under nitrogen is not an indispensable condition for the attainment of accurate data. Data obtained by us for the "intradex" preparation are in complete agreement with literature data [4] (Table).

In order to determine the number of 1,6-bonds and other bonds in the mannan molecule, we used the results of the periodate oxidation of a purified mannan preparation under the following experimental conditions. One hundred milligrams of mannan was treated with 40 ml of acetate buffer at pH 3.6, and 10 ml of 0.15 M $NaIO_4$; the volume of the reaction mixture was increased to 100 ml with bidistilled water. A periodate solution mixed with the buffer solution in the same proportions was kept as a control. The experimental solution and the control were both kept in the dark at room temperature. The determination of the amount of periodate consumed and formic acid produced was carried out on 5 ml aliquots.

Determination of the amount of periodate consumed. Five milliliters of the reaction mixture was treated with 10 ml of saturated sodium bicarbonate solution and 5 ml of 0.1 N Na_2AsO_3. The mixture was kept in the dark for 15 min. Then, 1 ml of 20% KI solution and a few drops of starch solution were added to the mixture, and the excess of arsenite was estimated by titration with a 0.1 N iodine solution. The difference between titration data of control solution and experimental mixture corresponded to the amount of $NaIO_4$ consumed (in ml of 0.1 N iodine).

Determination of the formic acid formed. Five milliliters of the reaction mixture was treated with 0.2 ml of freshly distilled ethylene glycol. The mixture was allowed to stand for 1 hr in the dark. Then, formic acid was titrated against 0.01 N NaOH with phenolphthalein indicator. The difference between the titration values of experimental solution and control gave the amount of HCOOH formed (in ml of 0.01 N NaOH).

The results of the investigations were the following:

Substance	Time of oxidation in hours				
	24	48	72	96	120
Formic acid produced (in moles w. r. to mannose residue)	0.52	0.55	0.62	0.62	0.62
$NaIO_4$ absorbed (in moles w. r. to the mannose residue)	1.68	1.62	1.62	1.68	1.82

Calculation based on the above-mentioned data shows that the different types of bonds in the mannan molecule are distributed as follows:

	48 hr	72 hr	96 hr
Number of 1,6-bonds	55%	62%	62%
Number of 1,2- and 1,4-bonds	52%	38%	44%
Number of other bonds	None	None	None

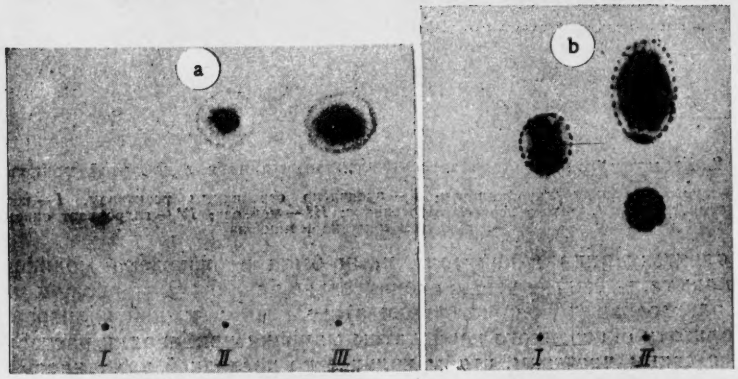

Fig. 1. Chromatograms of the hydrolyzate of oxidized mannan. A) Revealed with aniline hydrophthalate; B) revealed with the silver reagent. I) hydrolyzate; II) control; III) mannose.

The results obtained proved 1, 3-bonds were absent in the mannan molecule. In order to confirm this observation, we undertook further studies aiming at the elucidation of the nature of the periodate oxidation products of mannan by the method of paper chromatography.

Twenty milliliters of the solution containing the oxidation products of mannan with $NaIO_4$ (in the presence of the buffer, and in its absence) was treated with 1 ml of freshly distilled ethylene glycol, and the mixture was kept for 1 hr in the dark. In the control experiment, 20 ml of $NaIO_4$ solution of the same concentration (from the control flask) was kept in the dark for 1 hr after addition of 1 ml of ethylene glycol; then, 20 mg of mannan was added to the solution. Then, both solutions were freed from excess of ethylene glycol and its reaction products with sodium periodate by dialysis against running distilled water for 24 hr. The solutions were evaporated to dryness in porcelain dishes on a water bath, and the residues dissolved in 1 ml of 1 N H_2SO_4 and hydrolyzed in an ampul on a boiling water bath for 9 hr. The contents of the ampuls were neutralized (litmus) with dry $Ba(OH)_2$ and centrifuged. The neutralized hydrolyzate (experiment and control) was placed (0.05 ml amounts) on a strip of chromatographic paper. Elution was carried with butyl-acetic mixture. A marker sample of mannose (0.025 ml of 2% solution) was placed on the same chromatogram.

Experiments with omission of the dialysis step were carried out simultaneously. The other steps were carried out in the same manner. Results were consistent.

Spraying the chromatogram with aniline hydrophthalate as recommended by Partridge [5] on the level corresponding to the hydrolyzate of mannan previously oxidized with periodate, did not reveal any spot. The control displayed a well defined spot on the level of mannose (Fig. 1, A). The same results were obtained when the amount of hydrolyzate placed on the paper was increased. Spraying of a duplicate chromatogram with the silver reagent recommended by Trevelyan [6] along the line corresponding to the hydrolyzate of mannan oxidized with periodate, revealed a spot situated below the mannose spot in the control (Fig. 1, B). Beside the mannose spot, there was a small spot on the control line corresponding to the oligosaccharide usually found among the hydrolysis products of mannan [7]. Although this spot, as revealed by the silver reagent on the line of the hydrolyzate of the periodate-oxidized mannan, did not coincide with either a mannose spot, or the oligosaccharide spot in the control, we carried out a modification of the experiment in order to exclude mannose and the given oligosaccharide as sources for this spot. Thus, to a neutralized hydrolyzate of periodate-oxidized mannan we added a solution of mannose in such a way as to have different mannose: mannan ratios in a given volume of hydrolyzate (from 1 : 32 to 1 : 7). The mixture thus prepared, the hydrolyzate without addition of mannose, the control solution, and a mannose solution were then placed side by side on the starting line of the chromatogram. Spraying with aniline dihydrophthalate reagent after elution with butyl-acetic mixture led to the following results: on the line on the mixture, as well as on the line of the control, there was one spot at the level of mannose. On the line of the hydrolyzate without added mannose, there was no such spot (Fig. 2, A).

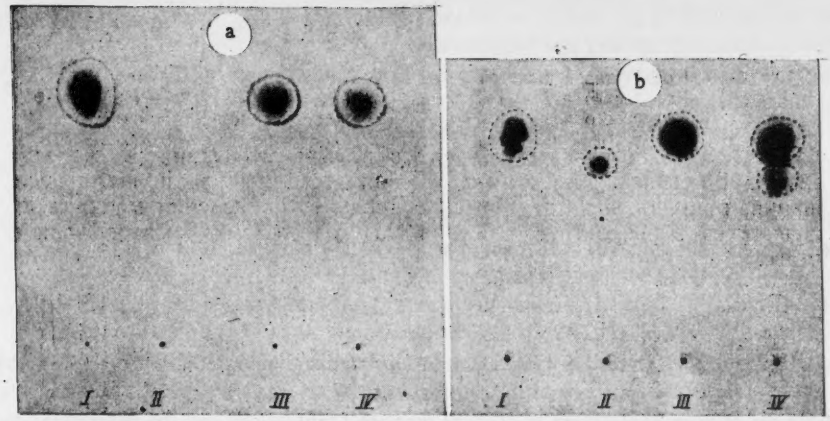

Fig. 2. Chromatograms of the hydrolyzate of oxidized mannan after the addition of mannose. A) Revealed with aniline hydrophthalate; B) revealed with the silver reagent. I) Control; II) hydrolyzate of periodate-oxidized mannan; III) mannose; IV) hydrolyzate of periodate-oxidized mannan with added mannose.

A second (duplicate) chromatogram was eluted with the butyl-acetic mixture twice. When it was sprayed with the silver reagent, the following results were obtained : on the line of the mixture there were two spots: one, on the level of mannose, and the other, a weaker one, on the same level as the spot detected in the hydrolyzate without added mannose (Fig. 2, B).

Therefore, the reducing substance of the hydrolyzate which is revealed by spraying with the silver reagent (a small spot below the mannose spot) is not due to mannose.

Thus, it was not possible to detect mannose among the hydrolysis products of periodate-oxidized mannan. This proves the absence of mannose residues linked in 1,3-position in the molecule of mannan—a result which confirms the experimental data obtained by estimating the amounts of periodate consumed and of formic acid produced during the periodate oxidation of mannan.

SUMMARY

Mannose residues in the mannan molecule of diphtheria bacteria are linked with one another by 1,6-; 1,2-; and [or] 1,4-bonds. The 1,6-bonds predominate.

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A RADIOMETRIC METHOD FOR THE QUANTITATIVE ASSAY OF HIPPURIC ACID IN URINE

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The ability of the liver to synthesize hippuric acid from benzoic acid and glycine was used by Quick for the functional diagnosis of liver in the presence of sodium benzoate [1, 2]. At the present time, Quick's test and its known modifications are of wide application in clinical practice [3-5]. However, the determination of hippuric acid by Quick's method is inaccurate, as it depends on the pH of the urine, and also on the temperature and the amount of water in which the hippuric acid to be titrated is dissolved [6].

We are proposing now a radiometric method for the assay of hippuric acid in urine which is a modification of Quick's test based on the method of isotopic dilution [7] with the use of C^{14}-labelled hippuric acid.

EXPERIMENTAL PART AND DISCUSSION

The principle of isotopic dilution is known if one adds to a mixture of various chemical substances a known amount (m) of a certain radioactive substance with a specific activity SA_1, then, the specific activity of this compound in the mixture will decrease in proportion to the initial concentration of the same (but not radioactive) substance in the mixture. Thus, if one can extract from the mixture a certain amount of the substance under investigation and determine its specific activity (SA_2), then one can calculate from the decrease in specific activity the degree of dilution of the radioactive compound with the same, but not radioactive species. This would allow to determine the amount of the non-radioactive species (x) in the original mixture, formula (I) being used:

$$x = \frac{(SA_1 - SA_2) \cdot m}{SA_2} \qquad (I)$$

Therefore, formula (I) can be used for the determination of hippuric acid in urine, after introduction of sodium benzoate in the organism as proposed by Quick, thus:

$$Q = \frac{\frac{V}{v}\left(\frac{SA_1 - SA_2}{SA_2}\right) \cdot m \cdot 0{,}804 \cdot 100}{4000}, \qquad (II)$$

where Q is the hippuric acid content in urine expressed as a percentage w.r. to the sodium benzoate introduced in the organism; V, the volume of a 4-hr diuresis; v, the volume of urine taken for analysis; m, the amount of C^{14}-labelled hippuric acid (in mg) added to the sample; 0.804, the conversion factor of hippuric acid into the equivalent amount of sodium benzoate [8]; SA_1, the specific activity of C^{14}-labelled hippuric acid added to the sample; SA_2, the specific activity of the C^{14}-labelled hippuric acid isolated from the sample (if identical procedure is adhered to in all assays, the values of SA_1 and SA_2 in formula (II) can be replaced by the corresponding activities in order to simplify the calculations); 100 is the factor introduced for obtaining the results as a percentage; 4,000, the amount of sodium benzoate given to the patient (in mg). When m = 100 mg, formula (II) can be further simplified to:

$$Q = 2{,}01 \frac{V}{v}\left(\frac{SA_1}{SA_2} - 1\right). \qquad (IIa)$$

Reagents. 1-C^{14}-Labelled hippuric acid was prepared by a biosynthetic method. A horse was fed through a tube, on an empty stomach, with 4 g of sodium 1-C^{14}-benzoate dissolved in 100 ml of water with a total activity of 270 μC and the tube was carefully washed with 500 ml of warm water. Then, the urine of the animal was collected for 4 hr. By addition of 2 N NaOH, the pH of the urine was raised to 8-8.5 (indicator paper used), and the

TABLE 1. Assay of Hippuric Acid in a Standard Solution by the Radiometric Method

Amt. of hippuric acid taken (mg)	Amt. of hippuric acid taken (mg)	Error of the method
100	106	+6
100	103	+3
100	97	−3
100	105	+5
100	105	+5
75	75	—
75	75	—
75	80	+6
Mean arithmetic deviation (%)		2,7

mixture was filtered. The filtered urine was then treated with non-radioactive hippuric acid so as to have 4 g of this acid per liter of the urine. The mixture thus obtained was treated (with stirring) with 2 N NaOH solution until all hippuric acid had dissolved, and with NaCl (300 g/liter) which dissolved upon warming to 50°. The labelled hippuric acid was precipitated from this mixture by acidification with concentrated hydrochloric acid until acid to Congo red, and cooled to 2-3° overnight. The crystals of C^{14}-hippuric acid thus obtained were filtered off, and dissolved on the filter in a minimum amount of 1 N NaOH solution. Purified hippuric acid was reprecipitated from this solution in the same manner as described above. Finally, the crystals of C^{14}-hippuric acid were separated from the mother liquor by filtration on a sintered Jena glass filter No. 3, and washed with water. The recrystallization process was then repeated once more. Twice-recrystallized carefully washed C^{14}-hippuric acid was dried to constant weight in a thermostat at 38-40°.

Thirty milligrams of radioactive hippuric acid gave 1200-1300 cpm on a target with a diameter of 18 mm, at a 10 mm distance from a plane counter (MST-17).

The sample of C^{14}-hippuric acid thus obtained was used to prepare a standard solution: 2.5 g of this acid was dissolved in 50 ml of 1 N NaOH. One milliliter of this standard solution contained 50 mg of C^{14}-hippuric acid.

Procedure for assay. Four hours after the patient was fed with sodium benzoate, the volume of urine produced was measured, and if its amount was not exactly 200, 300, or 400 ml, it was diluted with distilled water to the corresponding minimum volume. By taking into consideration the eventual dilution, 1/20th of the initial volume of previously filtered urine* was poured into a Hagherdorn beaker, and 2 ml of the standard solution of C^{14}-hippuric acid was added. After thorough mixing, the solution was acidified with concentrated hydrochloric acid (Congo red) and kept for 20 min in an ice-bath. The crystals of C^{14}-hippuric acid which separated out were isolated from their mother liquor by filtration through the sintered glass filter No. 3, and washed on the filter with three 1 ml portions of ice-cold distilled water, and two 0.5 ml portions of ether. The crystals thus washed were dried on the filter with

TABLE 2. Assay of Hippuric Acid in Urine by the Radiometric Method

Hippuric acid assayed in urine after addition of C^{14}-acid (mg)	C^{14}-Hippuric acid added to the urine (mg)	Hippuric acid assayed (mg)	Error w.r. to the hippuric acid added (%)	Hippuric acid assayed in urine after addition of C^{14}-acid (mg)	C^{14}-hippuric acid added to the urine (mg)	Hippuric acid assayed (mg)	Error w.r. to the hippuric acid added (%)
173	200	368	−3	124	200	312	−6
173	300	466	−2	112	100	211	−1
117	100	219	+2	115	100	219	+4
117	200	329	+6	105	100	208	+3
206	200	402	−1,5	144	100	250	+6
115	50	166	+2	165	100	271	+5
174	150	337	+8	244	200	442	−1
123	150	277	+2	211	200	411	—
124	100	224	—	67	200	259	−4
				227	150	381	+2
Mean arithmetic deviation,%							1,3

*In the case when urine contained albumin, the latter was precipitated by the method of Weichselbaum and Probstein [9].

TABLE 3. Comparative Data Given by Quick's Method as Modified by Nikitin, and by the Radiometric Method

Quick's method as modified by Nikitin		Radiometric method		Difference, (%)	Quick method as modified by Nikitin		Radiometric method		Difference, (%)
amt. of sodium benzoate					amt. of sodium benzoate				
g	% w.r. to the benzoate administered	g	% w.r. to the benzoate administered		g	% w.r. to the benzoate administered	g	% w.r. to the benzoate administered	
1,880	47	1,978	49,2	— 5	2,933	73,3	3,168	79,2	— 7,4
2,860	71,5	3,184	79,6	—10	1,435	35,9	1,608	40,2	—10,8
2,860	71,5	2,959	73,9	— 3,4	3,326	83,1	3,200	80,0	+ 3,8
3,242	81	3,345	83,6	— 3	3,278	81,9	3,762	94,0	—12,9
3,393	84,8	3,441	86,0	— 1,4	2,762	69	2,871	71,7	— 3,8
3,266	81,6	3,393	84,8	— 4	3,000	75	3,044	76,1	+ 1,5
3,182	79,5	3,58[illegible]	89,6	—11,3	0,696	17,4	0,646	16,1	+ 7,7
1,100	27,5	1,447	36,2	—24					
Mean arithmetic deviation, %									

the help of a water pump and current of air for 2 hr. The dried radioactive preparation was used to make two 30 mg aliquots on targets for radioactive assay. The aliquots were treated with 0.1 ml of 96% alcohol, and the whole mass was rubbed, and evenly distributed on the surface of the target with the help of a thin glass rod. The targets were then dried in a thermostat at 38-40° for 30 min.

Preparation of a standard sample. Four milliliters of the standard solution was treated with 10 ml of water, and the resulting solution was submitted to the same treatment as in the case of the experimental samples. The assay of radioactivity of the standard (SA_1) and experimental (SA_2) samples was carried out in a plane counter (MST-17) connected to a B-2 radiometer with a statistical reliability of 2-3% [7]. The standard preparations were kept in a desiccator over $CaCl_2$, and thus, they could be used over a long period of time.

Example of calculation. Volume of urine, 200 ml; volume of the aliquot, 10 ml. Mean activity of the standard preparations, 1,205 cpm. Mean activity of preparations assayed, 405 cpm. By introducing these values in equation (IIa), one obtains the amount of sodium benzoate isolated as hippuric acid from the urine, in % of the dose administered to the patient:

$$Q = 2{,}01\,\frac{200}{10}\left(\frac{1205}{405} - 1\right) = 79{,}2\%$$

The radiometric method was used to assay hippuric acid in the standard solution and in the sample of urine. The data obtained are given in Tables 1 and 2. The method described (Table 1) allows to determine the amount of hippuric acid in the standard solution with an accuracy of −3% to + 6%; the mean arithmetic deviation was 2.7%.

In the case of hippuric acid added to urine (Table 2), the amplitude of the deviations was greater, viz., from −6% to + 8%, the mean arithmetic deviation being 1.3%. During a simultaneous assay of hippuric acid in urine by the proposed radiometric method and by Quick's method as modified by Nikitin [8], we noticed (Table 3) that in the greatest number of experiments (12 out of 15), Quick's method gave lower values than the radiometric method. The discrepancy between the two methods varied from −1.4% to −24%, and the mean arithmetic deviation of the results obtained by Quick's method (as modified by Nikitin), as compared with the radiometric method was not higher than −5.6%.

In Quick's method, in distinction from the radiometric method, the results of the assays depend on many factors which are sometimes difficult to take into account, or to control, viz., the completeness of precipitation of hippuric acid from urine, the pH of urine, the temperature and volume of water in which hippuric acid is dissolved during titration, and the carefulness with which the crystals of hippuric acid are washed free of hydrochloric or sulfuric acid. Therefore, one can consider that more accurate values in hippuric acid assay have been obtained by the application of our radiometric method.

SUMMARY

The radiometric method allows to assay quantitatively the amount of hippuric acid present in urine.

The radiometric method for the assay of hippuric acid in urine is more accurate than Quick's method.

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All abbreviations of periodicals in the above bibliography are letter-by-letter transliterations of the abbreviations as given in the original Russian journal. ***Some or all of this periodical literature may well be available in English translation.*** **A complete list of the cover-to-cover English translations appears at the back of this issue.**

A STUDY OF THE DECOMPOSITION PRODUCTS OF MONOMYCIN

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In a previous communication we have described a method for isolating the new antibiotic monomycin and have shown its difference from neomycin and kanamycin [1]. In the present work we give the results of a chemical study of the products of decomposition of monomycin and their identification.

EXPERIMENTAL

Methanolysis of monomycin. Fifty g of monomycin base and 750 ml of 0.65 N HCl in absolute methanol were heated under reflux for 33.5 hr in a glycerol bath at 80°. We obtained 30 g of a crystalline precipitate, insoluble in methanol which was called monamine. Monamine was recrystallized from 4 N HCl and then from aqueous methanol. The monamine crystals were colorless plates The neutralization equivalent of monamine was 160; molecular weight 342; $[\alpha]^{20}D = 85°$; N – 9.43%; Cl – 23 3%.

The monamine, like the antibiotic had a positive ninhydrin reaction, but in distinction from monomycin, did not give a Bial reaction. The monamine contained three amino groups, shown by the method of obtaining partial derivatives with dinitrofluorobenzene for monamine and further products of its decomposition and later paper electrophoresis of these derivatives [2, 3]. The monamine had a very weak antibacterial activity, much less than neamine, obtained from neomycin [4].

From the methanol solution, purified by carbon, after isolation of monamine we obtained component II. The methanol solution was evaporated 18-20 fold in a vacuum without heating and component II was precipitated with acetone. We thus obtained 29 g of strongly colored product which was purified successively on a column with neutral aluminum oxide and wofatite E.

Study showed that component II contained 2 amino groups, gave clear Bial and ninhydrin reactions, but was not developed on a paper chromatogram with a solution of aniline phthalate.

The neutralization equivalent of component II was 217, molecular weight 387; N, 6.47%. On the chromatogram (Fig. 1) there was a reciprocal arrangement of these components and the initial monomycin.

Hydrolysis of monamide. Four g of monamine and 50 ml of 6 N HCl were heated in a sealed ampule at 100° for 14 hr. For isolation of the products formed in the hydrolysis we carried out distribution chromatography on a column with 175 g of cellulose (diameter of the column 4.2 cm, height 58 cm). Eight ml fractions were collected. The solvent was the upper phase mixture of butanol-CH_3COOH-H_2O (4 : 1 : 5). From fractions 155-226 we isolated a chromatographically pure component, 650 mg. It was crystallized from ethanol. Under these conditions hydrolysis of monamine does not result in its complete splitting; but with increased time of hydrolysis there begins to be decomposed also the products of its hydrolysis. The neutralization equivalent of the crystalline product is 188; number of amino groups, 1; N, 6.45%. The isolated crystalline component was identified chromatographically in 8 systems of solvents with a glucosamine standard, and also by the method of Stoffyn and Jeanloz [5]. This method consists in treatment of glucosamine samples deposited on paper with a solution of ninhydrin in ethanol with 4% pyridine and keeping the paper with the sample over 50% pyridine at 80° for 3 hr. On deamination the glucosamine forms arabinose which after drying of the chromatogram is chromatographed in the solvent system butanol-ethanol water (4: 1: 1) with a standard and is developed with a solution of aniline phthalate (Fig. 2).

Hydrolysis of monomycin. Twenty g of monomycin hydrochloride was heated in a sealed ampule with 6 N HCl at 140° for 18 hr. The hydrolyzate was purified with activated charcoal. Only one product was detected

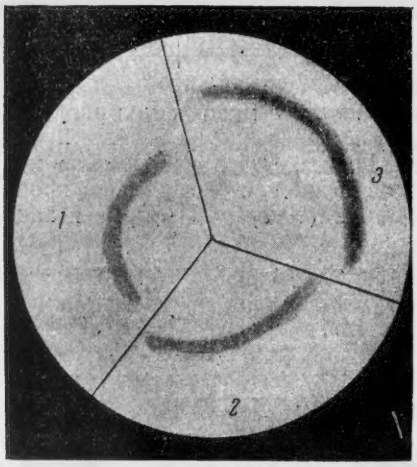


Fig. 1. Chromatogram of the products of methanolysis of monomycin (monamine and component II). Solvent: butanol-pyridine-CH_3COOH-H_2O (15 : 10 : 3 : 12). 1) Monomycin; 2) monamine; 3) component II.

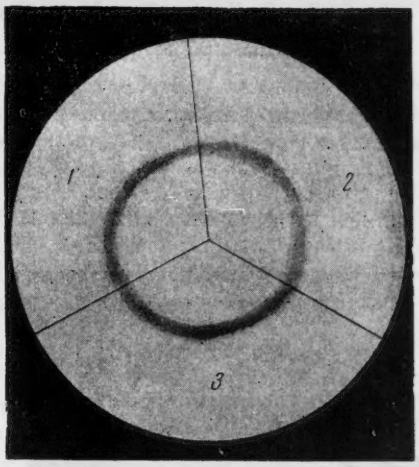


Fig. 2. Chromatogram of deaminated glucosamino (standard and sample from monomycin) and developed arabinose from the deamination. Solvent: butanol-ethanol-H_2O (4 : 1 : 1). 1) Deaminated D-glucosamine; 2) deaminated glucosamine from monamine; 3) arabinose.

chromatographically in the hydrolyzate; it was adsorbed on cationite KB4-P2 in the sodium form. Elution was carried out with 1 N NH_4OH. Crystallization of the base from 4 N HCl gave a hydrochloride which was recrystallized from 80% ethanol. We obtained 3 g of substance. The neutralization equivalent was 84; number of amino group 2; N 11.47%.

An analogous product was found in neomycin and kanamycin [6, 7] and was isolated earlier by us from the hydrolyzate of kolimycin (neomycin) by hydrolysis of this antibiotic with 6 N HCl at 140°; this product was purified on cationite KB4-P2 and identified as deoxystreptamine by the nitrogen of the hydrochloride, the acetyl and benzoyl derivatives (table) and also by the presence of two amino groups.

Deoxystreptamine obtained from monomycin was identified chromatographically in 8 systems of solvents with the deoxystreptamine isolated from kolimycin.

In Fig. 3 we give a chromatogram of glucosamine and deoxystreptamine with standards.

Deamination of monomycin base. Three g of monomycin was dissolved in 24 ml of water, pH 5, 2.3 g of sodium nitrite was added, and after 2 hr, 1.8 ml of glacial acetic acid was added. After an hour the solution was neutralized with $Ba(OH)_2$, filtered and successively passed through a column with cationite KB4-P2 (10 g) in the H^+ form, and the anionite EDE-10 in the OH^- form. The solution was evaporated and the deamination product was precipitated with acetone. By an analogous method we obtained the deaminated component II. Hydrolysis of deaminated antibiotic and component II was carried out with 1 N HCl for 30 min at 100°.

Obtaining the N-acetal derivative of monomycin. Five g of monomycin base, 20 ml of water, 10 g of EDE-10 in the OH^- form, 10 g of $(CH_3CO)_2O$ in portions was shaken for 2 hr, then the solution was passed through a column with resin KB4-P2 to remove residues of the antibiotic which had not been acetylated; it was evaporated and precipitated with acetone. We thus obtained 6.2 g of substance. Acetylated component II was obtained in an analogous way. Hydrolysis of the acetylated product was carried out with 1 N HCl for 60 min at 100°.

Chromatography. The conditions of methanolysis of monomycin and hydrolysis of monamine and component II were controlled chromatographically. Chromatography was carried out on a disk of Leningrad chromatographic paper for rapid filtration in a Petri dish (diameter 10 cm). The sample was deposited at the center of a circle with a 1 cm diameter. The solvent was poured into the bottom of the dish. The lower end of a wick of string fiber which passed through the center of the paper dipped into the solvent in the dish, on the edge of which was placed the paper, covered with a cover. The concentration of hydrolyzate and standard which we used was 10-20 μg. The solvent

Analytical Data (N, %) in Identification of Deoxystreptamine

Preparation	Deoxystreptamine	
	from kolimycin	from monomycin
Hydrochloride	11.62 (theoretical 11.93)	11.47
N-acetal derivative	11.65 (theoretical 11.67)	
N-benzoyl derivative	7.37 (theoretical 7.57)	

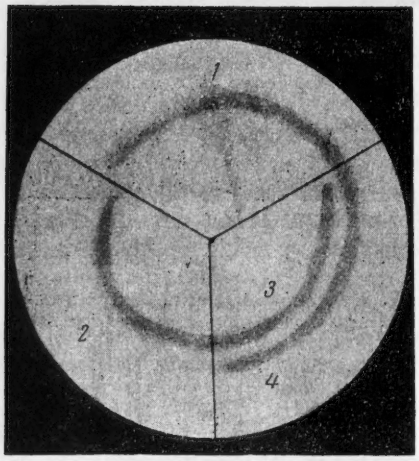


Fig. 3. Chromatogram of hydrolysis product of monamine (glucosamine and deoxystreptamine with standards). Solvent: butanol-pyridine-CH_3COOH-H_2O (15 : 10 : 3 : 12). 1) Glucosamine from monamine; 2) deoxystreptamine from monamine; 3) deoxystreptamine from kolimycin; 4) D-glucosamine.

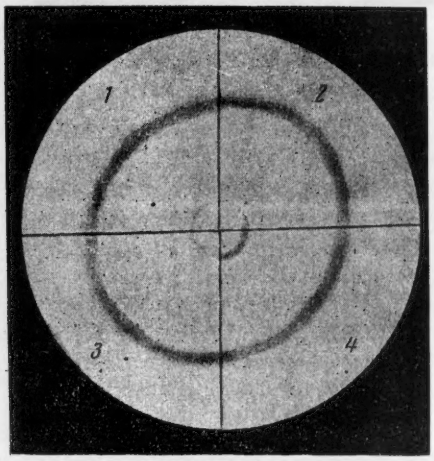


Fig. 4. Chromatogram of hydrolyzate of N-benzoylated, N-acetylated, and deaminated monomycin and ribose. Solvent: butanol-ethanol-H_2O (13 : 8 : 4). 1) Ribose; 2) hydrolyzate of benzoyl derivative of component II; 3) hydrolyzate of deaminated component II; 4) hydrolyzate of acetyl derivative of component II.

was passed for one hour. The chromatogram was produced in the following systems: 1) butanol-pyridine-CH_3COOH-H_2O (15 : 10 : 3 : 12); 2) butanol-CH_3COOH-H_2O (4 : 1 : 5); 3) butanol-ethanol-H_2O (4 : 1 : 1); 4) butanol-ethanol-H_2O (13 : 8 : 4) (Fig. 4).

The chromatograms were dried at 70-80° to complete removal of the solvent, dipped in the developer (0.2% acetone solution of ninhydrin or a solution of aniline hydrophthalate), and then heated for 5 min at 105° The ninhydrin color was deepened by dipping the chromatogram in a 1% acetone solution of $Cu(NO_3)_2$.

DISCUSSION

The antibiotic monomycin when heated in absolute methanol with HCl is split into crystalline monamine and component II.

The bond between these components is most labile and sensitive to acids. However, the antibiotic is stable to the action of alkali which indicates the presence of a glucosidal link between monamine and component II. It is interesting to see that monamine, like neamine, has a weak antibacterial activity. When monamine is heated with 6 N HCl it is hydrolyzed into glucosamine and deoxystreptamine, the presence of which was shown by chromatography and analytically after preliminary isolation. Although component II gives a positive Bial reaction, it is not

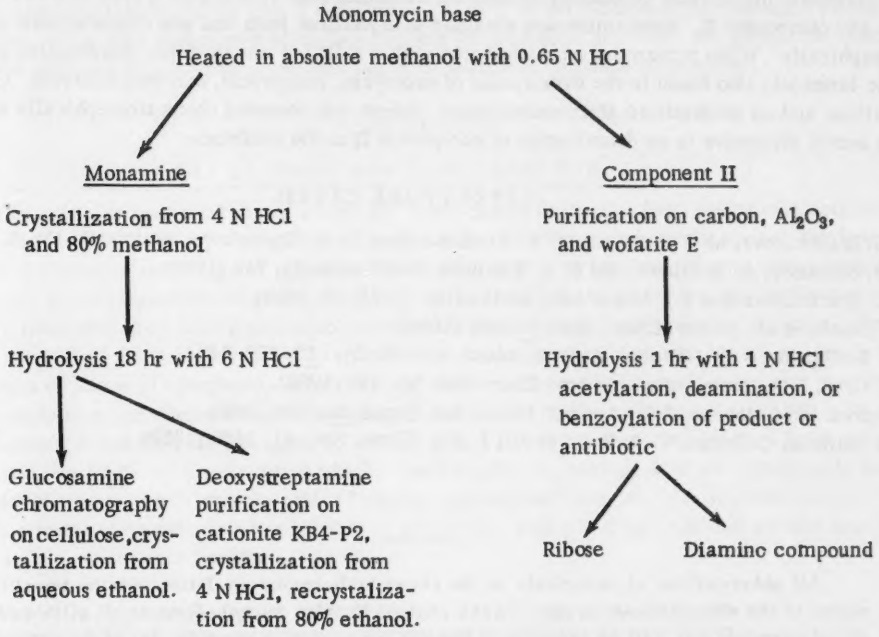
developed on paper by a solution of aniline phthalate, evidently because of the "shielded" glucosidal group, protected by the methyl group.

Attempts to detect pentoses chromatographically after weak acid hydrolysis were not successful. However, component II after 30 min hydrolysis with 1 N HCl was converted into a compound which gave a reaction with aniline phthalate, was colored by ninhydrin, and occupied a different position on the chromatogram as compared to component II.

Evidently under these conditions there was a splitting of the glucoside with liberation of the glucosidal hydroxyl of the pentose and appearance of the reaction with aniline phthalate. On further hydrolysis, component II (in a study of different conditions of hydrolysis) could not give any pentose chromatographically, and under more severe conditions (10 hr with 6 N HCl) the Bial reaction vanished completely, which indicates destruction of the pentose.

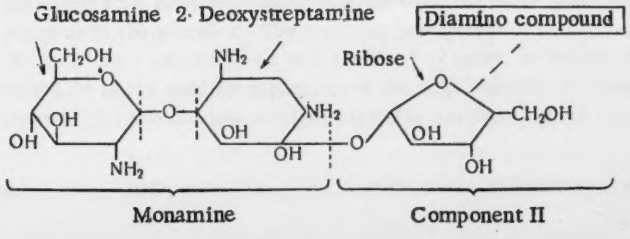
However, if we hydrolyze the acetyl or benzoyl derivatives of monomycin or component II or hydrolyze monomycin or component II with deamination, then we can easily show chromatographically the presence of ribose in the hydrolyzate. In this connection we can suggest that the presence of a free amino group in some way hinders the appearance of ribose. Identification of ribose was carried out chromatographically in eight systems of solvents (Fig. 4).

Separation of the products of splitting of monomycin under different conditions of degradation can be expressed as follows:


Monomycin base
Heated in absolute methanol with 0.65 N HCl
Monamine
Component II
Crystallization from 4 N HCl and 80% methanol
Purification on carbon, Al_2O_3, and wofatite E
Hydrolysis 18 hr with 6 N HCl
Hydrolysis 1 hr with 1 N HCl acetylation, deamination, or benzoylation of product or antibiotic
Glucosamine chromatography on cellulose, crystallization from aqueous ethanol.
Deoxystreptamine purification on cationite KB4-P2, crystallization from 4 N HCl, recrystalization from 80% ethanol.
Ribose
Diamino compound

Thus, chemical study of monomycin showed that this antibiotic contains 4 constituent components, deoxystreptamine, glucosamine, ribose, and an amino compound with two amino groups.

On the basis of the results obtained with chemical study of monomycin we can suggest a structure of one of the fragments of this antibiotic, monamine, and a plan of the structure of monomycin as follows:



At present time it is still difficult to say how the hydroxyl groups take part in the combination of glucosamine with deoxystreptamine, what is the diaminocompound, and how the ribose is combined. Since monomycin does not give the aniline phthalate reaction, it is evident that the glycosidal hydroxyl of ribose takes part in the compound with another component, evidently with deoxystreptamine. This bond is also very labile in methanolysis.

It is important to remark that the separation in a Craig apparatus of the fully substituted dinitrophenyl derivative of monomycin shows the nonhomogeneity of this antibiotic. Monomycin consists mainly of three variants, present in diffferent amounts. The amount of the variant which occurs in the greatest quantity is above 70%. Evidently the quantitative ratio of the variants can be changed in biosynthesis. The presence of variants is also confirmed by paper chromatography of the N-acetal derivative of monomycin. In this case also we find three variants.

At the time of completing this work there appeared a paper in the Journal of the American Chemical Society on the structure of the antibiotic paromomycin [8]. This antibiotic, according to the report of the authors, contains glucosamine, deoxystreptamine, ribose, and 6,3-diaminoglucose, combined with each other in a definite order through hydroxyl oxygen. Whether monomycin is identical with paromomycin or differs in the diamino sugar, cannot be stated at the present time. However, these antibiotics are very similar in their chemical structure, and their comparison is of undoubted interest.

SUMMARY

The antibiotic monomycin on heating in absolute methanol with 0.65 N HCl is split into two components, monamine and component II. Monoamine was obtained in crystalline form and was characterized analytically and chromatographically. When monamine was hydrolyzed with 6 N HCl it was split into glucosamine and deoxystreptamine. The latter was also found in the hydrolyzate of neomycin, kanamycin, and paromomycin. Component II contained ribose and an unidentified diaminocompound. Ribose was detected chromatographically only on hydrolysis of the acetyl derivative or on deamination of component II or the antibiotic.

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THE METABOLIC ACTIVITY OF COMPONENTS OF NUCLEAR SAP*

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In our previous work we described the existence in nuclear sap of a material obtained by salt extraction of isolated nuclei, RNP particles of two types ("microsomes" and "ultramicrosomes" of nuclear sap) [1]. We also described some of their chemical and metabolic properties. Finally, we showed in special experiments that RNP granules and the pH 5 fraction obtained from nuclear sap were actually of nuclear origin and not products of cytoplasmic impurities. At the same time and independently of us a similar type of investigation was carried out by Frenster and others in the laboratory of Mirsky [2], and partly by Kit [3].

In the present communication we give a detailed description of the method of fractionation of nuclear sap, a study of the nature of the RNA component of nuclear sap and its metabolic activity. We discuss the question of the importance of the different components of nuclear sap in intranuclear biosynthetic processes.

METHODS

Isolation and Fractionation of Materials of Nuclear Sap

As object of study we used the liver of adult rats. We isolated the nuclei from liver tissue obtained from 6-10 animals by the two stage sucrose-glycerophosphate method [4]. For precipitation of the nuclei, the homogenate was centrifuged in a refrigerated centrifuge of type MSE-SS-25 at 40,000 g for 20 min. The resulting nuclei were extracted with 25 ml of a mixture of 0.14 M KCl, 0.001 M $MgCl_2$, and 0.01 M tris-buffer, pH 7.2 by shaking for 10 min. The nuclei were precipitated at 2000 g and again extracted with new portions of the same mixture. The free extract contained the material of the nuclear sap [1, 5]. To remove the larger particles, which were evidently fragments of nuclei and parts of scraps of cytoplasm, we centrifuged the extract for 15 min at 14,000 g and in most of the experiments the precipitate was discarded. The supernatant liquid was further centrifuged at 56,000 g for one hour in a MSE SS-25 centrifuge and then for two hours at 100,000 g in a UTsP-1 ultracentrafuge (in a large rotor). As a result we obtained two fractions of precipitate which, conditionally, by analogy with the cytoplasmic fractions sedimented out under the same conditions, we called "nuclear microsomes" and "nuclear ultramicrosomes." The supernatant liquid in a series of experiments was brought to pH 5.0-5.1 with 1 N CH_3COOH and we thus separated at pH 5 a fraction and a final supernatant fraction.

Isolation and Characteristics of RNA from Nuclear Sap Fractions

The isolation of RNA from nuclear sap was carried out by the phenol method [6]. The precipitates of "microsomes" and "ultramicrosomes" from nuclear sap were suspended by means of a Potter homogenizer in 5-10 ml of buffered 0.14 M NaCl (0.01 M tris, pH 7.2). To the suspension was added an equal volume of phenol; the mixture was shaken, centrifuged, and the aqueous phase was collected. Extraction with buffered 0.14 M NaCl was repeated and then the combined extracts were repeatedly treated with phenol, pH 6. RNA was precipitated from the water phase by alcohol. For obtaining RNA from the supernatant fraction, the latter was treated with phenol, pH 6, and further treatment took place as in the granules. The resulting precipitate of RNA (yield about 400-600 μg RNA in each fraction, with 60-70 g of liver) was dissolved in 0.3-0.4 ml of water, an equal volume of 4 M NaCl was added, and the mixture stood overnight in the cold for separation of the high polymer fraction [7, 8]. The high polymer RNA was collected by centrifuging and the low polymer material was precipitated from the supernatant liquid by

*We have used the abbreviations: RNP, ribonucleoprotein; DNP, deoxyribonucleoprotein; SA, specific radioactivity.

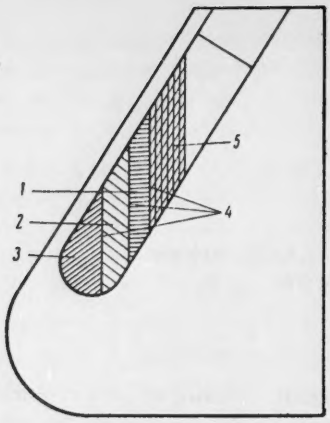

Fig. 1. Plan for packing layers in ultracentrifuge preparations in starch. 1, 2, 3) Starch layers; 4) layers of filter paper; 5) RNA solution.

alcohol. For removal of excess salt and phenol, all the fractions of RNA were once more reprecipitated by alcohol.

For further analysis of RNA in the nuclear sap fractions we used the method of preparative ultracentrifuging on starch worked out by one of us, using the small rotor in an UTsP-1 ultracentrifuge. The tube contained three layers of packed starch separated by layers of filter paper. The liquid phase contained buffered 0.14 M NaCl and for greater stability a layer of sucrose; in the first layer from the bottom of the tube 0.8 M, in the second layer 0.4 M and in the third layer, 0.2 M. We dissolved 0.5-1 mg of RNA in 1 ml of buffered 0.14 M NaCl and layered it on the surface of the starch (Fig. 1). Centrifuging lasted 70 min at 50,000 rpm (180,000 g) at 18°. Then the RNA was collected separately from the supernatant liquid and the three starch layers, and its amount was measured spectrophotometrically. Comparison was carried out with standard preparations of high and low polymer RNA isolated from cells of Ehrlich ascitic tumors which were ultracentrifuged at the same time as the objects studied. The molecular weight of such standard preparations is well known from the literature ($0.5\text{-}2 \cdot 10^6$ and $2\text{-}3 \cdot 10^4$, respectively for the high and low polymer RNA). A detailed description of the method will be given in the following communication.

Study of the Uptake of P^{32} in the RNA Fractions

The experiments on the uptake of P^{32} were carried out as follows: rats weighing about 200 g were injected intraperitoneally with 0.5 μ curie/g of weight of P^{32}. The animals were killed after 30 min, 1 hr, and 144 hr after the injection; the nuclei were separated and the nuclear sap and its fractions were isolated. The high and low polymer RNA were separated as described above from the microsomes, ultramicrosomes, and supernatant liquid. Also, we removed from the residue which was insoluble in water and phenol the "nonextractable" RNA by treating with 10% NaCl for one hour on the boiling water bath. In the experiments with P^{32}, RNA was also obtained for analysis from other cells fractions. From the supernatant liquid of the homogenized tissue after removal of the nuclei we collected a portion, diluted it with seven volumes of water and removed the remaining nuclei at 2000 g for 10 min, then added NaCl to a concentration of 0.14 M, and isolated the high polymer, low polymer, and nonextractable RNA of the cytoplasm by the phenol method [10].

The nuclear residue obtained after removal of the nuclear sap was washed once more with the same mixture and then extracted with 2 M NaCl to remove DNP. The acid protein was separated from the DNP by centrifuging at 60,000 g for 30 min. As a result, the DNP solution was entirely clear, but contained a small amount of RNA. The RNA along with the DNA and part of the protein was precipitated from the solution by alcohol and then extracted with 10% NaCl at 100°. The residue after removal of the DNP was suspended in buffered 0.14 M NaCl and then RNA was isolated by treatment with phenol at pH 6. Practically all the RNA liberated from the acid protein fraction was shown to be the high polymer form in salt fractionation, that is, it was precipitated by 2 M NaCl. All the RNA fractions which we obtained were washed, hydrolyzed with 0.5 N KOH, purified with activated charcoal (Norite), and their SA was determined as described in previous work [10].

Since in the work of Frenster and others [2] there was shown to be a metabolic inequality of the RNA obtained in successive extractions of the nuclei of calf thymus by tris-buffer, pH 7.1, we decided to repeat these experiments on our material. For this purpose, 30 min after injection of the animal with P^{32} we isolated the nuclei from rat livers and extracted one of the portions of the nuclei three times by our method, collecting each extract separately, and another portion, three times by the method of Frenster et al. [2] with 0.1 M tris-buffer, pH 7.1, in a Waring blendor, also collecting each fraction separately. We removed the RNA from all the extracts with hot 10% NaCl and determined its SA.

Study of the Uptake of Labeled Amino Acids in the Protein Fraction of Nuclear Sap

Labeled amino acids (glycine-C^{14} and tyrosine-C^{14}) were injected intraperitoneally into rats at 20-30,000 counts/g of weight and with periods of 15 min to 64 hr before killing. Nuclear sap fractions were obtained from the

isolated nuclei. Also after 9 fold dilution of the cytoplasmic fractions with the salt mixture used for extraction of the nuclear sap, we obtained by differential centrifuging the mitochondria, microsomes, ultramicrosomes, and supernatant liquid [11]. The washing and determination of SA in the resulting protein fractions were described in a previous work [12].

RESULTS AND DISCUSSION

Characteristics of RNA from Nuclear Sap Fractions

For the characteristics of the RNA isolated from the "microsomes" and "ultramicrosomes" and supernatant fraction of the nuclear sap we chiefly used the method of phenol and salt fractionation. The results (in percent of total RNA of nuclear sap) were as follows:

Component of nuclear sap	Highpolymer RNA	Low polymer RNA	Nonextractable RNA
"Microsomes"	25.4	1.9	6.3
"Ultramicrosomes"	29.8	4.4	5.1
Supernatant fraction	3.7	21.6	1.8

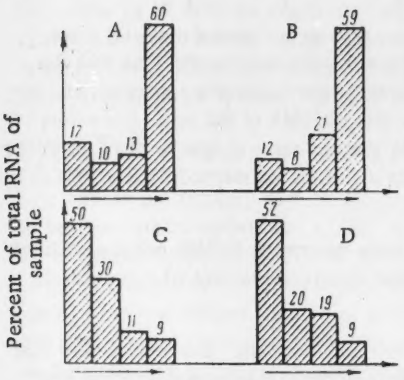

Fig. 2. Sedimentogram of RNA of nuclear sap and standard preparations of high and low polymer RNA. A) RNA of ribonucleoprotein granules of nuclear sap; B) high polymer RNA of Ehrlich tumor (standard preparation); C) RNA of supernatant fraction of nuclear sap; D) low polymer RNA of Ehrlich tumor (standard preparation). Arrows show the direction of sedimentation. For experimental conditions, see text.

It follows from the results that practically all the RNA of the RNP particles of nuclear sap is precipitated by 2 M NaCl, that is, the high polymer, while the greater part of the RNA of the supernatant fraction remains in solution, that is, the low polymer. This is true for nuclear sap both from rat livers and calf thymus. Also all the fractions of nuclear sap contained a small amount of RNA not extracted by phenol. Parallel carrying out of salt fractionation of RNA isolated from the acid protein fraction showed that it too was high polymer RNA, precipitated by 2 M NaCl. Salt fractionation permitted separation of high and low polymer RNA, but for further verification of the degree of polymerization of RNA we used the method of starch ultracentrifuging (Fig. 2). We analyzed the RNA fractions of the sum of the granule fractions and the supernatant fraction of nuclear sap, and also standard preparations of high and low polymer RNA. In the ultracentrifuge treatment the standard high polymer or the RNA of nuclear sap granules showed about 60% RNA in the lower starch layer and only about 15% remained in the starting upper water layer. The reverse situation occurred in the ultracentrifuge preparations of standard low polymer RNA and RNA from the supernatant nuclear sap fraction. Here the greater part (about 50%) of the RNA remained in the upper water layer, and only a very small part (about 10%) reached the lower starch layer. The sedimentograms of the standard RNA preparations and the corresponding preparations of nuclear sap RNA were almost exactly the same.

These results show that the "microsomes" and "ultramicrosomes" of nuclear sap contain high polymer RNA, and the supernatant fraction of nuclear sap contain low polymer RNA.

Uptake of P^{32} in the RNA Fractions of Nuclear Sap

There is very great interest in studying the uptake of P^{32} in the early sap after injection of the precursor (after 30-60 min) since if there is a reciprocal relationship between the early fraction RNA for precursor-product, then the sharpest differences in their SA will be shown in just this early period. The results are given in Table 1.

If we consider the uptake of P^{32} by high polymer RNA of different cell structures, then it seems that in the early period the most active labeled material is taken up in the RNA of the chromosome-nucleolus apparatus, then in RNA of the RNP particles of nuclear sap, and then very weakly in the high polymer RNA of the cytoplasm. It is

TABLE 1. Uptake of P^{32} in Vivo in Different Types of RNA from Cell Fractions of Rat Liver

Cell structure	Subfraction	RNA type	SA, counts/min/μg RNA			
			30 min	30 min	1 hr	144 hr
Cytoplasm		High polymer	0.03	0.041	0.057	4.4
		Low polymer	0.26	0.49	0.36	4.4
		Nonextractable	0.52	–	1.08	3.9
Nuclear sap	Microsomes	High polymer	2.92	2.2	3.42	3.6
	Ultramicrosomes	High polymer	4.52	4.9	5.0	3.5
	Supernatant fraction	Low polymer	5.82	7.8	6.6	3.5
Chromosome-nucleolus apparatus	DNP fraction	–	2.95	3.5	2.0	3.6
	Acid protein	High polymer	7.8	10.4	8.5	3.5

also a fact that the high polymer RNA from the "ultramicrosomes of nuclear sap take up the labeled material more intensively than the high polymer RNA of the nuclear sap "microsomes." These results confirm the idea that the place of synthesis of cell high polymer RNA found in the ribosomes is the chromosome-nucleolus apparatus, and before they enter the cytoplasm the ribosomes pass through the nuclear sap, so that the RNA of the latter has a very high SA, not much less than the SA of the nucleolus RNA. It is interesting to note that the ratio of uptake of P^{32} in microsome RNA to uptake of P^{32} in ultramicrosome RNA of nuclear sap is the same as for the corresponding fractions in the cytoplasm [13]. The significance of this fact is still uncertain.

The uptake of P^{32} in unextractable RNA of the cytoplasm greatly surpasses the uptake in high polymer extractable RNA, while for nuclear sap these values are very close. The uptake in the unextractable RNA of cytoplasm is markedly lower than in any of the fractions from nuclear RNA.

There is great interest in the question of the place of biosynthesis of low polymer RNA. Our results show that the uptake of P^{32} in low polymer RNA of nuclear sap is about 20 times higher than the uptake in cytoplasmic low polymer RNA This makes very probable the idea that not only the biosynthesis of the high polymer RNA of the ribosomes, but also the synthesis of low polymer RNA, occurs in the cell nuclei. In another study from our laboratory [10] we also obtained some evidence for a connection in the synthesis of low polymer RNA and the chromosome-nucleolus apparatus. In its SA the low polymer RNA of nuclear sap yields only to the RNA of the acid protein fraction.

The amount of RNA not extracted by phenol in all the fractions of nuclear sap and also the low polymer RNA in the microsomes and the high polymer RNA in the supernatant fraction are very small (see table 1) and so we could not determine their specific activities with sufficient accuracy (the error was ± 30-50%). On the whole, the uptake in nonextractable RNA was about the same as in the high polymer RNA of the corresponding fraction of nuclear sap. The specific activity of the low polymer RNA of the ultramicrosomes was the same as of the low polymer RNA of the supernatant fraction, and the high polymer RNA of the supernatant fraction was the same as for the high polymer RNA of the ultramicrosomes. Thus, the small amount of high polymer RNA which precipitated in the supernatant fraction and the low polymer RNA which was in the ultramicrosomes were found as cross impurities of these fractions in the ultracentrifuging.

The uptake of P^{32} in RNA of the DNP fraction was markedly less than the uptake in the RNA of the acid protein (RNA of the ribosome nucleonemes). Evidently we are dealing here with a particular RNA fraction and not with impurities in the DNP fraction from the acid protein RNA fraction. This question requires individual study.

An investigation of the uptake of P^{32} in later periods after injection of the isotope showed an equalizing of the SA of different fractions, and the SA of the RNA from cytoplasm was somewhat higher than in the nuclear RNA.

TABLE 2. Comparison of Results of Extraction of Nuclear Sap by the Method of the Authors and by the Method of Mirsky et al. [2]

No. of extracts	Mixture by [1]		Tris-buffer by [2]	
	amt., %	SA	amt., %	SA
1	70	3,1	57	3,7
2	25	4,3	32	3,9
3	5	(2,6)*	11	3,8

*Because of the small amount of RNA in this fraction, the values obtained for the SA are not significant.

We compared our method of isolating the material in nuclear sap and the method of Frenster et al. [2] for obtaining RNP particles of the nuclei. As these authors reported, using a 0.1 M tris-buffer, pH 7.1, the first extract from the isolated nuclei contained RNA with a very low SA, in the second extract the SA of the RNA was higher, and the third, much higher. These experiments were run on nuclei from calf thymus glands and incubation with the precursor of the nucleic acid was carried out in vitro. In work with rat liver nuclei on the uptake of P^{32} in vivo (in the course of 30 min) we could not find any inhomogeneity of RNA in SA in successive extractions by tris-buffer (Table 2). The value of SA for RNA from nuclear sap obtained from the same nuclei by the solvents which we usually used did not differ from the SA of RNA extracted by this tris-buffer. Thus, in work with nuclear sap from rat livers there is no need for special studies of successive extracts from the nuclei.

Uptake of Labeled Amino Acids in the Protein Fraction of Nuclear Sap

Some preliminary data on the uptake of labeled amino acids in the protein fraction of nuclear sap were given in previous communications [1, 12]. A series of supplementary experiments gave confirmation and some more details for the earlier conclusions (Table 3). In the early period the amino acids are taken up most actively in the proteins of the "microsomes" of nuclear sap. The SA of this fraction is about 1.5 times higher than the SA of the "ultramicrosomes" and supernatant fraction of nuclear sap. The uptake of labeled amino acids in the proteins of the granular fraction of the cytoplasm is more intense than in the nuclear sap, while the supernatant fraction of cytoplasm and nuclear sap take up labeled materials to the same extent. It is interesting to note that in both cytoplasm and nuclear sap the uptake in the microsome fraction is higher than in the ultramicrosomes. On the other hand, as shown above, the RNA of the ultramicrosomes takes up labeled material more intensively than the RNA of the microsomes.

In the later period the highest SA is found in the supernatant fraction of cytoplasm and nuclear sap, while in the granules it is considerable less. There is practically no difference between the microsomes and the ultramicrosomes in this period.

The question of how significant are the different structures and fractions of nuclear sap in total protein and nucleoprotein balance of the cells is still not entirely clear, although some ideas can be expressed on this subject.

In the cell structures of nonmitotic tissues there are evidently two general types of synthesis of macromolecules. These are the biosynthesis of ribosomes and the biosynthesis of soluble proteins of cell nuclei and ribosomes. The formation of ribosomes is reflected in the uptake of labeled precursors in the high polymer RNA and amino acids in the protein, and the synthesis of soluble protein only in the uptake of amino acids in the protein. The synthesis of high polymer RNA and the protein part of the ribosomes occurs obviously in the chromosome-nucleolus apparatus of the cells [10, 12], whence they pass through the nuclear sap into the cytoplasm. It is interesting to note that in accord with the data of salt fractionation, the RNA of the nucleonemes of the chromosome-nucleolus apparatus consists of high polymer RNA. The high uptake of P^{32} in the RNA of the RNP particles of nuclear sap and especially in the RNA of the "ultramicrosomes" of nuclear sap shows that they also are probably "transit ribosomes," passing from the nucleus into the cytoplasm. Our results also show that along with the ribosomes there is evidently synthesized also the low polymer RNA which also passes through the nuclear sap into the cytoplasm. This fact may be of great significance in connection with the theory of Hoagland on the adaptor role of low polymer RNA in the biosynthesis of proteins on a matrix of high polymer RNA [9]. Other synthetic processes evidently occur in the nuclear sap itself:

TABLE 3. Uptake of Labeled Amino Acids in the Protein Fraction of Nuclear Sap

Fraction	Specific activity												
	15 min						30 min		1 hr	16 hr		64 hr	
	I	II	I	II	I	II	I	II	I	I	II	I	II
Microsomes	156	372	112	307	165	300	337	550	—	440	440	250	200
Ultramicrosomes	75	280	63	122	107	143	258	270	—	500	404	260	185
Microsomes + ultramicrosomes	—	—	—	—	—	—	—	—	307	—	—	—	—
Supernatant fraction	90	92	75	74	120	118	153	134	371	780	700	380	255

Note: I– Nuclear sap; II–cytoplasm.

the biosynthesis of soluble proteins of the cell nuclei. Actually, the nuclear sap contains the same basic component systems required for the biosynthesis of proteins as does the cytoplasm: ribosomes in the composition of the fractions of "microsomes" and "ultramicrosomes" which contain high polymer RNA, soluble low polymer RNA and pH 5 enzyme. In work from the laboratories of Mirsky [2, 13] and Rendi [14] it was shown that in isolated RNP granules of the nuclei there can occur uptake of amino acids into protein with activation of the amino acids; their transfer to soluble RNA and inclusion in ribosomes evidently takes place in the same way as in cytoplasm [2, 13, 15]. Our results indicate that in the early period higher uptake of amino acids occurs in the protein of the nuclear "microsomes." Here, although the radioactivity of the "microsome" RNA is considerably lower than that of the "ultramicrosome" RNA, the uptake of labeled material in the "microsome" proteins is 1.5 times higher. This disproportion is evidence that the uptake of amino acids in the protein granules of nuclear sap reflects, besides the new formation of ribosomes, the synthesis in them of special nuclear soluble proteins, and a leading role in this process is evidently played by the nuclear "microsomes."

Before final judgement on the question of the type of biosynthesis in cell nuclei we must consider that in mitotic tissue there is still a third type of biosynthesis of macromolecules, the premitotic doubling of the DNP complex.

SUMMARY

We have given a detailed description of the isolation from nuclear sap of two types of ribonucleoprotein particles: "microsomes" and "ultramicrosomes" of nuclear sap. Using phenol and salt fractionation and ultracentrifuging on starch we have shown that the ribonucleoprotein particles of nuclear sap contain high polymer RNA and the supernatant fraction of nuclear sap contains low polymer RNA. The latter can be precipitated in the composition of the pH 5 fraction. By the salt test, the RNA in the acid protein of the nuclei is also high polymer.

The uptake of P^{32} in the early period in the high polymer RNA occurs most actively in the chromosome-nucleolus apparatus (RNA of acid protein), then the "ultramicrosomes" and "microsomes" of nuclear sap. In the cytoplasmic high polymer RNA the uptake of P^{32} in the early period is several hundred times lower. The radioactivity of the low polymer RNA of nuclear sap is 20 times above the radioactivity of the low polymer RNA of cytoplasm. Evidently the synthesis of both, the high polymer RNA of the ribosomes and the low polymer RNA, bound to the chromosome-nucleolus apparatus and their transfer to the cytoplasm occur through the nuclear sap.

Labeled amino acids in the early period are most actively taken up in the proteins of the nuclear sap "microsomes." In the later period the supernatant fraction has the greatest activity. The uptake in the proteins of components of nuclear sap evidently reflects the formation and transfer of ribosomes from the nucleus into the cytoplasm, and also the synthesis of soluble nuclear proteins in the ribosomes of nuclear sap.

We have discussed the question of the chief types of protein biosynthesis and nucleoprotein biosynthesis in cell nuclei and the role of nuclear sap in these processes.

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THE NEUTRAL FRACTION OF LOW MOLECULAR PEPSIN-PEPTIDES FROM EQUINE HEMOGLOBIN

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In the last two years there have appeared a considerable number of communications concerning the peptides of hemoglobin which are formed by its acid hydrolysis or as a result of the action of enzymes such as trypsin on hemoglobin [1]. A survey of this work has appeared in our recent review [2].

Little attention has been devoted up to now to the study of pepsin-peptides. Besides the older work of Brown [3] and the work of Oppel' [4] there has recently appeared the work of Braunitzer and co-workers [5, 6] on combined digestion by pepsin and trypsin.

Most of the work devoted to study of the peptides obtained from hemoglobin was carried out with human hemoglobin. Many data have been obtained on the structure of the anomalous peptides formed from pathological forms of human hemoglobin [2]. There is comparatively little work on the splitting of animal hemoglobins. The school of Sorm [7, 8] and Măsiar [9-11] reported a number of studies on peptides from the hemoglobin of horses, swine, cats, and rats.

The present work is concerned with a continuation of our previous studies and is devoted to a study of the peptides obtained in the splitting of equine hemoglobin by pepsin. We describe the experiments with the neutral fraction.

METHODS

Digestion. Crystalline horse serum, prepared under laboratory conditions, was dissolved in water and mixed with HCl to obtain 0.1 N HCl. Such a solution, 120 ml containing 1.42% hemoglobin, was digested for 23 hr at 38.7° with pepsin under a layer of toluene.

Obtaining the low molecular fraction. Pepsin hydrolysis was stopped by placing the flask for 5 min in a boiling water bath. After cooling the hydrolyzate, the undigested protein was precipitated with the high molecular peptides by addition of an equal volume of 20% TCA with shaking. After settling for 2.5 hr, the liquid was filtered through an ash free filter. The TCA was removed from the filtrate, which contained the lower peptides, by repeated extraction with ether, and the traces of ether were removed by heating on a boiling water bath. The volume of liquid was brought to 240 ml with water. Determination of nitrogen (by a modified Conway method) showed 62% of the protein had been converted to the fraction of low molecular peptides.

Fractionation by electrodialysis. The solution, neutralized with ammonia, was fractionated into neutral, acid, and basic peptides by electrodialysis in a five-chamber Solov'ev apparatus with a chrom-gelatine membrane, platinum anode, and copper cathode [12]. During the electrodialysis the liquid in the middle chamber was continually neutralized by addition of ammonia. At the end of the electrodialysis the collected fractions were evaporated in a vacuum and kept for analysis in a desiccator in dry form.

In the present work we describe experiments with the peptides of the neutral fraction.

Separation of the peptides. A small part of the fraction of neutral peptides was dissolved in a minimum volume of a mixture of ethyl alcohol and 0.1 N HCl (2 : 1). Several microliters of this solution were placed at one of the angles of a two dimensional chromatogram with dimensions 24 × 24 cm (Leningrad "rapid" chromatographic paper). We used two mixtures as solvents: 1) n-butanol-ethanol-NH_3-H_2O (20 : 60 : 7 : 13), and 2) n-butanol-CH_3COOH-H_2O)(25 : 6 : 25). Passage of solvents was stopped after the front had advanced 18 cm. The dried chromatogram was sprayed with 0.02% ninhydrin in n-butanol and kept in a dark place at room temperature until the

next day. The peptide spots observed on the chromatogram were circled with a pencil. We studied 8-10 chromatograms in parallel and the corresponding spots on them were cut out and treated together.

Ten of the 25 peptides studied in the present work gave well defined spots on the two dimensional chromatogram, the amino acid analysis of which showed not more than six different amino acids in their composition. The other peptides gave a combination of spots which had to be submitted to further separation. This was the case especially with the peptides with less chromatographic mobility.

For this, the corresponding ninhydrin, stained portions of several two dimensional chromatograms were cut out and eluted with a mixture of ethanol and 0.1 N HCl (2 : 1); the eluate was evaporated on a steam bath and finally dried in a desiccator. The resulting small dry residue of mixture of peptides was again dissolved in three drops of the mixture of ethanol and 0.1 N HCl and deposited as a band on a narrow, long (60 cm) strip of chromatographic paper through which mixture (2) was passed 2-3 times Before repeating the passage of solvent, the chromatogram was air dried. After the end of the chromatography an edge was cut from the dried chromatogram and was treated with 0.2% ninhydrin in n-butanol. On the next day the borders of the colored spots were noted on the strip, and the corresponding spots, not colored by ninhydrin, were cut from the main part of the chromatographic strip. Elution was carried out with the mixture of ethanol and 0.1 N HCl. The eluate was evaporated on a steam bath and dried in a desiccator.

Amino acid analysis. The small, dry peptide residue was dissolved in two drops of 6 N HCl and the solution was quantitatively sucked up in a glass capillary which was sealed off. The capillary was placed in a drying oven heated to 110°. After hydrolysis, which was continued for 20-22 hr, the contents of the capillary were transfered quantitatively to a small porcelain crucible, diluted with distilled water, and evaporated dry in a desiccator over NaOH. For more complete removal of the HCl, the residue was repeatedly dissolved in water and evaporated in a desiccator The peptide hydrolyzate was deposited on a 60 cm long strip of chromatographic paper. At the side of the drop of hydrolyzate was placed a drop of a mixture of standard amino acids. The latter, to the number of 18, were placed in three columns so that amino acids which had similar R_f values would not cover each other. After triple development of the chromatogram from mixture (2) and coloring with 0.2% ninhdrin, we identified the amino acids of the peptides studied.

A number of peptides were separated and studied repeatedly (from 2 to 7 times). There were nine such peptides. Since in repeated analyses their composition was identical, this permitted us to separate and analyze the remaining 16 peptides once.

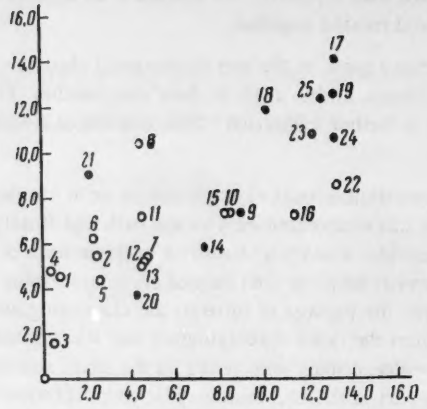
RESULTS AND DISCUSSION

In the table we give the results of analysis of 25 peptides isolated in our experiments. Some of the peptides were isolated and analyzed repeatedly (up to 7 times). Most of the peptides (16) were analyzed once. Fifteen peptides were separated in repeated one dimensional chromatographic spots, cut from a two dimensional chromatogram. Ten peptides were sufficiently clearly separated on the two dimensional chromatogram, so that analysis did not require further chromatography. In the table all the peptides are arranged in groups according to the number of amino acids contained in them. Since quantitative determination of the latter was not carried out, we cannot speak of the number of amino acid residues in each peptide and the molecular weight of the peptides can be calculated only as the minimum molecular weight.

The absence of experiments to show the successive order of amino acids in the peptides cannot exclude the possibility of existence of some isomers. Such a possibility is indicated by the fact that in a number of experiments, peptides with the same composition had several different R_f values.

Taking account of these limitations in the table and figure which show schematically the distribution of the peptides on the two dimensional chromatogram, the results can be generalized as follows. In the neutral fraction of low molecular peptides, from pepsin, there are no peptides which contain histidine, and very few (one) peptides which contain arginine (No. 15) and cysteine (No. 16).

As to cysteine, this is explained by the fact that even after three day digestion the cysteine pepsin-peptides which are obtained are rather high molecular [3]; they contain from 9 to 14 different amino acids. In our experiments with equine hemoglobin we found one cysteine pentapeptide (No. 16) which differed markedly in composition from the particular pentapeptide isolated from human hemoglobin [3]. It also differed from the cysteine pentapeptide isolated from equine hemoglobin by direct acid hydrolysis [9].



Scheme of two dimensional chromatogram of neutral fraction from low molecular pepsin-peptides of equine hemoglobin. Vertical, solvent n-butanol-ethanol-NH_3-H_2O (20 : 60 : : 7 : 13). Horizontal, solvent n-butanol-CH_3COOH-H_2O (25 : 6 : 25). Distribution from starting line (cm) on abscissa and ordinate. Solid circles, peptides eluted directly from the two dimensional chromatogram; hollow circles, peptides repeatedly separated on one dimensional chromatogram.

In the work of Masiar and Smolnicky [10] it was shown that the trypsin-peptides of equine hemoglobin, which contained histidine, behaved as cations in electrophoresis. This evidently explains the absence of the histidine pepsin-peptides in the neutral fraction of the pepsin digestion. The same thing occurs in the case of the arginine-peptides.

The presence of glycine in the pepsin-peptides at all times is a characteristic of them. We remarked on this effect previously [1]. Considering the considerable content of glycine in the molecule of equine hemoglobin [13], equal to 48 residues per 68,000 g of hemoglobin, this fact should not cause surprise. There are many peptides which contain serine, tyrosine, lysine, leucine, and glutamic acid. The appearance of these peptides is quite regular, since the peptide bond with these amino acids is easily ruptured by pepsin [16].

The peptides which contain proline (Nos. 9 and 14) evidently come from different parts of the hemoglobin chain. Peptide No. 14 is very similar to a fragment of the β-chain of human hemoglobin. If we assume the possibility of replacement of one glutamic acid residue by serine, analogous to its replacement by glycine in pathological human G hemoglobin [14], then we can ascribe hypothetically for the β-chain of equine hemoglobin the succession -leucine-threonine-proline-glutamic acid-serine- or -leucine-threonine-proline-serine-glutamic acid instead of the succession-leucine-threonine-proline-glutamic acid-glutamic acid found in human hemoglobin A. If this succession of amino acids actually occurs in

Amino Acid Composition of Neutral Pepsin-Peptides

Peptide No.	Amino acid composition	Peptide No.	Amino acid composition	Peptide No.	Amino acid composition	Peptide No.	Amino acid composition
1*	Glycine, aspartic, lysine	3	Alanine, glutamic, aspartic, lysine	12	Alanine, glycine, serine, glutamic, lysine	18	Leucine, phenylalanine, tyrosine, glycine, serine
2*	Glycine, glutamic, lysine	4	Glycine, serine, aspartic, lysine	13	Valine, glycine, serine, glutamic, lysine	19*	Leucine, valine, tyrosine, glycine, serine
		5*	Threonine, glycine, aspartic, lysine	14	Leucine, proline, threonine, glutamic, serine	20*	Valine, tyrosine, glycine, serine, glutamic lysine
		6*	Glycine, serine, glutamic, lysine	15	Alanine, threonine, glycine, serine, arginine	21*	Leucine, tyrosine, glycine, serine, glutamic, lysine
		7	Alanine, threonine, glutamic, lysine	16	Leucine, valine, glycine, aspartic, cysteine	22	Leucine, tyrosine, glycine, glutamic, aspartic, lysine
		8	Valine, tyrosine, glycine, serine	17	Leucine, valine, alanine, glycine, serine	23	Leucine, valine, tyrosine, glycine, glutamic, lysine
		9	Leucine, proline, glutamic, glycine			24*	Leucine, valine, tyrosine, threonine, glycine, serine
		10	Leucine, glycine, serine, glutamic			25	Leucine, phenylalanine, tyrosine, threonine, glycine, serine
		11*	Leucine, tyrosine, glycine, serine				

*Peptides isolated and analyzed more than once.

equine hemoglobin, then the N-terminal of its β-chain would seem to be valine-glutamic acid-leucine-threonine-proline-glutamic acid-serine. As was shown previously [2], three N-terminal amino acids of the β-chain in equine hemolgobin are-valine-glutamic acid-leucine-. Rupture of the bond glutamic acid-leucine by pepsin, just as with the bond serine-lysine, is entirely possible.

To a known degree there is a similarity between the composition of peptide No. 17 and the peptide obtained by pepsin splitting of the trypsin-peptide from the β-chain of human hemoglobin A, isolated and described by Hilschmann and Braunitzer [6]; peptide No. 17 from equine hemoglobin has leucine, valine, alanine, glycine, serine, and the peptide from human hemoglobin has leucine, valine, $alanine_2$, serine, threonine. We cannot now say whether the same portions have been obtained from both hemoglobins.

Comparing the peptides, which contain phenylalanine, which were obtained in the present work: leucine, phenylalanine, tyrosine, glycine, serine (No. 18), and leucine, phenylalanine, tyrosine, threonine, glycine, serine (No. 25) with the phenylalanine-peptides isolated by the action of γ-rays on equine hemoglobin solutions [15], leucine, phenylalanine, glycine; leucine, phenylalanine, glycine, threonine; phenylalanine, glycine, threonine, tyrosine, and assuming that the latter three peptides are formed by rupture of the same portion of the polypeptide chain, we can describe a successive arrangement of the amino acids in the pepsin-peptide No. 25: Leucine-(phenylalanine, glycine)-threonine-tyrosine-serine- [1], or -serine-leucine- (phenylalanine, glycine)-threonine-tyrosine- [2].

The second of these arrangements seems more probable in view of the often noted serine in the N-terminal pepsin-peptides [16] obtained by splitting equine hemoglobin. On the other hand, tyrosine is an especially characteristic amino acid in C-terminal peptide bonds split by pepsin.

Peptide No. 18, in spite of its great similarity with peptide No. 25, evidently belongs to another portion of the polypeptide chain (chains ?). Reconstructing it from the peptides obtained in splitting hemoglobin by γ-rays would meet with difficulties in view of the threonine residue, which should be placed in the middle of the molecule.

Finally, the above attempts to explain the succession of amino acids in some pepsin-peptides on the basis of a comparison of their amino acid compositions and the compositions and structures of peptides given in the literature should be confirmed by direct determination of the succession of the amino acids in them.

We turn our attention to the similarity of the amino acid composition of peptide No. 23 in this study and peptide No. 12 of the previous one [1]. The difference lies only in the replacement of tyrosine (No. 23) by alanine (No. 12). Since the R_f values of these two amino acids differ very little in the mixture n-butanol-CH_3COOH-H_2O, and in the previous work chromatographic passage of the solvent took place only once, there remains the possibility of an error in method for solving the question of which of the two amino acids occurs here, raising the idea of the identity of both peptides. However, recognition of the identity is still difficult and there is a difference in R_f values for both peptides: 0.56 (No. 23) and 0.60 (No. 12) in the mixture n-butanol-ethanol-NH_3-H_2O, and 0.53 (No. 23) and 0.68 (No. 12) in the mixture n-butanol-CH_3COOH-H_2O.

Evidently the identity of the tripeptide glycine, glutamic acid, lysine found in the pepsin digestion of hemoglobin (peptide No. 2 of the present work, peptide of the preceding work [1]) is established, also, by its splitting under the influence of γ-rays [15].

We also compare the peptides No. 2 and No. 6, No. 8, No. 13, and No. 20, No. 11 and No. 21, No. 19 and No. 24 and consider them as fragments of the same portion of the polypeptide chain (chains ?). However, we abstain from considering such a hypothetical structure until more data have been accumulated on the succession of the amino acids in them.

Study of hemoglobin has been carried out very intensively in recent years. It has been established that equine hemoglobin, like many other hemoglobins (human, beef, sheep, goat), is not homogeneous, and consists of several hemoglobins [17]. Studies similar to ours reflect the results of analyses of the sum of these hemoglobins. On the other hand, methods recently worked out permit separation, and hence, also separate studies of the α- and β-chains of both human and equine hemoglobins [18]. A detailed investigation of both chains separately would give valuable information on hemoglobin structure.

SUMMARY

From the neutral fraction of low molecular pepsin-peptides of equine hemoglobin we have isolated and analyzed qualitatively the amino acid composition of 25 peptides. They contain from three to six different amino acids.

In the neutral fraction there is no histidine-peptide and few (only one) arginine-and cysteine-peptides. This fraction is rich in glycine, serine, tyrosine, lysine, leucine, and glutamic acid.

We have made an attempt to establish the succession of amino acids in three sections of the polypeptide chain of equine hemoglobin which here evidently includes the N-terminal peptides of the β-chain.

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A COMPARISON OF THE ENZYMATIC HYDROLYZATES OF NONSPECIFIC γ-GLOBULINS AND ANTIBODIES OF RABBITS

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A comparison of the chemical properties of nonspecific γ-globulins and antibodies, and also of the composition of separate antibodies is of great theoretical importance. Such a comparison could lead to solution of questions of the nature of the biological activity of antibodies and also of problems of the mechanism of their biosynthesis. In particular, on the basis of such comparisons, we could draw conclusions as to the acceptability of the views developed by Horowitz [1] and Pauling [2], according to which the antibody does not differ in its composition from the nonspecific γ-globulins, nor one antibody from another, but they differ only in the form of twisting of the polypeptide chain.

A comparison of the amino acid composition of antibodies and nonspecific γ-globulins carried out by a number of authors [3-5] did not show differences. Thus, for example, Smith and co-workers [5] showed that the amino acid composition of each of the rabbit antibodies against pneumococcus of types I, VII, VIII, and XIV was identical for the separate antibodies and the composition of the nonspecific γ-globulin. However, on the basis of these results, because of the deficiencies of the existing methods, it was not possible to draw an unconditional conclusion as to the complete absence of differences between the proteins which were compared.

It was not possible to draw definite conclusions as to the identity of antibodies and nonspecific γ-globulins in the study of their N-terminal amino acids. On the one hand, Porter [6], and McFadden and Smith [7] showed that different rabbit antibodies and nonspecific γ-globulin from rabbit had the same N-terminal amino acid, alanine; on the other hand, there are results which show a difference between nonspecific γ-globulin and antibodies. These results were obtained by Smith and McFadden [8], showing that horse antipneumococcus antibodies contained considerably more N-terminal alanine than horse nonspecific γ-globulin. The possibility of a difference in N-terminal amino acids in different antibodies was indirectly shown by the fact that in distinction from rabbit γ-globulin, the γ-globulin of other animals was very heterogeneous in respect to N-terminal amino acids: in beef γ-globulin, was 5; in horse γ-globulin, 7; and in human γ-globulin, 2 different N-terminal amino acids [4].

In recent years it has been shown that a comparison of the peptides formed by enzymatic hydrolysis of proteins permits demonstration of differences between proteins very similar in structure. This difference is shown by comparison of the electrophoresis-chromatograms obtained as a result of electrophoresis and later chromatography carried out successively in a mutually perpendicular direction (method of "finger printing" [9, 10]). Differences were thus shown between hemoglobin from the blood of healthy persons and hemoglobin from persons with sickle-cell anemia [9].

Since antibodies and nonspecific γ-globulins have very similar structures, then final solution of the question of the identity or difference of their molecules should be determined by the use of the electrophoresis-chromatography method. In view of the absence in the literature of such work, we took up the present study. We compared the enzymatic hydrolyzates of nonspecific γ-globulins and two pure rabbit antibodies isolated from the same serum: the antibody against human serum albumin (anti-HSA) and the antibody against equine serum albumin (anti-ESA). We carried out two sets of experiments: in the first series native protein was submitted to the action of proteolytic enzymes, and in the second, protein first denatured with TCA.

There is especial value for such a study in frequent comparison of the protein preparations. Since previously one of us [11-13] worked out a method for isolating pure antibodies by fixation of antigens on cellulose, we had the possibility of using antibody preparations for our study which were practically free from other protein impurities.

METHODS

Immunization of the animals. Each rabbit was immunized simultaneously with two proteins: HSA (electrophoretically pure preparation isolated by the alcohol method) and ESA (twice recrystallized protein. Immunization was carried out by the following scheme: in the course of three weeks the animals were injected every other day, intravenously (first week) with 1 mg, then (second week) with 2 mg, and finally (third week) with 3 mg of each protein. Then there was a break (2-6 months) and the animals were again injected with 50 mg of each of the proteins. After a week the animals were exsanguinated and the antibody content was determined by a somewhat modified Heidelberger method [14, 15].

Preparation of Materials

Isolation of pure antibodies. From each of the sera which we obtained we isolated the two antibodies: anti-HSA and anti-ESA. Isolation was carried out by an immunosorbent according to the method which we described previously [11-13]. The method consists in strongly fixing the protein used for immunization on cellulose powder by a halogen alkylate, N(m-nitrobenzyloxy)methylpyridinium chloride. The fixed protein retained its antigenic properties and the immunosorbent selectively held the corresponding antibody. Removal of the protein unbound with the immunosorbent was carried out by washing with 1% NaCl; and the antibody bound to the immunosorbent was split off by an acidified solution (1% NaCl, pH 3.2, or a phosphate-citric acid buffer, pH 3.2). As was shown before, the antibody isolated by this method from serum, first heated 40 min at 56°, (to decompose the complement) did not contain impurities of side proteins. The absence of impurities indicated by electrophoresis was also shown with respect to the antibody used in the present work.

Isolation of nonspecific γ-globulin from rabbits. The nonspecific γ-globulins were isolated from serum of nonimmunized rabbits by two methods: with rivanol [16] and by convection electrophoresis. Preparation of γ-globulins by the rivanol method was carried out by precipitation of all the residual serum proteins by addition of a triple volume of 0.4% rivanol solution. The γ-globulins which remained in solution were precipitated by making alkaline to a pH of 9.5. The resulting preparation was freed from admixtures of rivanol and further freed from protein impurities by electrophoresis on a starch block. The isolated preparation of γ-globulins was electrophoretically pure. An electrophoretically pure preparation of γ-globulins was also obtained in an apparatus for convection electrophoresis constructed by SKTB BFA. The isolated γ-globulins were lyophilized.

Preparation of the Materials for Hydrolysis

Dialysis and concentration of the proteins. Enzymatic hydrolysis of the proteins which we studied was carried out in an ammonia buffer pH 8.0; the concentration of the proteins being compared was made exactly equal, and salt side products were removed. For this purpose in the first series of experiments the protein was treated as follows: 1) It was precipitated by addition of an equal volume of saturated $(NH_4)_2SO_4$ solution, dialyzed against 0.2% NaCl and lyophilized. Before hydrolysis the lyophilized antibody was dissolved in 0.1 M solution of $(NH_4)_2CO_3$, pH 8.0 and dialyzed against the same solution. 2) It was concentrated under pressure in a cellophane bag lowered into 0.1 M $(NH_4)_2CO_3$. Under these conditions there occurred simultaneous concentration and dialysis. 3) The nonspecific γ-globulins were dissolved in 0.1 M $(NH_4)_2CO_3$ and dialyzed against the same buffer.

In the second series of experiments (enzymatic hydrolysis of denatured protein) the proteins were treated in another way: 1) To a solution of antibody was added an equal volume of 10% TCA. The precipitating antibody was separated by centrifuging. The TCA contained in the precipitate was removed by ether and the precipitate was dissolved in 0.1 M $(NH_4)_2CO_3$. 2) We prepared a solution of nonspecific γ-globulins of the same concentration as the concentration of the antibody used in the given experiment. Further treatment of the γ-globulin solution was carried out in the same way as the treatment of the antibody solution.

Enzymatic hydrolysis of antibody and nonspecific γ-globulin. Enzymatic hydrolysis was carried out under strictly standard conditions in an ammonia buffer (0.1 M $(NH_4)_2CO_3$, pH 8.0). The solutions of nonspecific

γ-globulins dialyzed against this buffer were diluted by it to a concentration corresponding to the concentration of the antibody solution (determined by the method of Lowry [17]) submitted to hydrolysis in the same experiment. Trypsin was added to the prepared solution, calculated at 0.2 mg of trypsin per 20 mg of protein, dissolved in 4 ml of ammonia buffer. Samples were incubated at 37° in a water thermostat with continuous rocking for two hours. After two hours we added to each sample 0.2 mg of chymotrypsin and incubation was continued for two more hours under these conditions.

At the end of the incubation the samples were lyophilized and for removal of $(NH_4)_2CO_3$ were placed for 5-7 days over P_2O_5 and NaOH in a vacuum desiccator.

Electrophoretic separation of antibody and nonspecific γ-globulin hydrolyzates. The lyophilized hydrolyzate was dissolved in distilled water and deposited on a paper strip (4 × 40 cm) dipping into a volatile buffer, pH 4.1, with the following composition: pyridine, 4%; CH_3COOH, 10%; and H_2O 86%. The strip with the deposited material was placed in an apparatus for electrophoresis under a layer of toluene. Separation was carried out at a potential gradient of 10 v and a current strength of 1.0-1.2 ma/cm for 3.5-5 hr. The electrophoretogram was developed with 0.5% solution of ninhydrin in acetone or cyano-black stain. The stained electrophoretogram was treated on an integrating densitometer constructed by SKTB BFA (with an OS-13 filter).

Electrophoresis-chromatographic study of the hydrolyzates from antibodies and nonspecific γ-globulins. Besides the electrophoretic separation of enzymatic hydrolyzates, in some of the experiments we carried out their separation by electrophoresis-chromatography, which in a number of cases permitted separation of the hydrolyzate with a larger amount of components.

This study was carried out by the method of "finger printing" [9, 10]. The material was deposited on a sheet of chromatographic paper (28 × 50 cm) impregnated with buffer solution, pH 5.6, of the following composition: pyridine 2%. CH_3COOH 0.5%, and H_2O 97.5%. Electrophoretic separation was carried out at 700-740 v and current strength 4.0-6.0 ma for 2.5 hr. At the end of electrophoresis, the sheet of paper was removed from the electrophoresis chamber and dried (to remove the buffer), using a current of air. Chromatography was carried out in such a way that the direction of the stream of solvent was perpendicular to the direction in which electrophoresis had been carried out. For chromatography (lasting 14 hr) we used the following system of solvents: butanol-H_2O-CH_3COOH (4 : 5 : 1). The dried electrophoretogram-chromatogram was developed with 0.5% solution of ninhydrin in acetone.

RESULTS

Study of Enzymatic Hydrolyzates of Native Nonspecific γ-Globulin and Antibodies

In the electrophoresis of the enzymatic hydrolyzate of rabbit nonspecific γ-globulins the chief mass of peptides under the conditions described above migrate from the place of deposition to the cation side and only two peptides shift from the point of deposition to the anode side. These two peptides are absent on the densitometric curves.

In Fig. 1,a we give curves for treatment on the densitometer of the electrophoretogram of the enzymatic hydrolyzate of nonspecific γ-globulins isolated by the rivanol method. The separate peptides are designated by Roman numerals in the order of decreasing rate of migration in the electric field. The picture presented in the figure is well reproduced in various experiments with nonspecific γ-globulins isolated by the rivanol method. The picture obtained in the experiments with nonspecific γ-globulins isolated by the method of electrophoretic convection was also very similar (Fig. 1 b).

For an explanation of the nature of the individual peptides, some of the electrophoretograms were colored, not with ninhydrin, but with acid cyano-black stain. It was shown that acid cyano-black stained only one fraction, VIII. Evidently this fraction consists of unsplit proteins or their large fragments. The other fractions are developed by ninhydrin and are not stained by cyano-black, and thus are smaller peptides. In several experiments for comparison we took the enzymatic hydrolyzates of nonspecific γ-globulins from horse serum. The peptide composition of the γ-globulins of horses was sufficiently close to the composition of γ-globulins of rabbits (Fig. 1 c) but we could see some differences between these proteins. Thus, for example, in the hydrolyzate from rabbit γ-globulin peak IX was always lower than X, and in the hydrolyzate from horse γ-globulin it was always much higher than its neighbor. Another difference in the horse γ-globulin hydrolyzate was the presence of an additional fraction II_2.

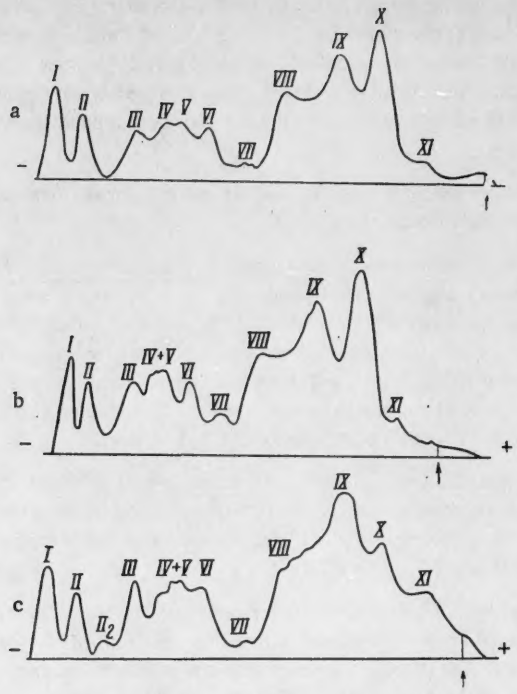

Fig. 1. Electrophoretic separation of enzymatic hydrolyzates of nonspecific γ-globulins of rabbit (a and b) and horse (c). a) Hydrolyzate of nonspecific γ-globulins isolated by the rivanol method, b) hydrolyzate of nonspecific γ-globulins of rabbits isolated by convection electrophoresis, c) hydrolyzate of nonspecific γ-globulins of the horse.

In the study of enzymatic hydrolyzates from rabbit antibodies we showed that some fractions of enzymatic hydrolyzates of the antibodies resembled the fractions of the hydrolyzates of nonspecific γ-globulins and could be designated by the same numerals (VIII-XI), but a number of peptides of rabbit antibody hydrolyzates differed from the peptides of rabbit nonspecific γ-globulin hydrolyzates.

On hydrolysis, the antibodies were split to a smaller number of fragments than the nonspecific γ-globulins. This was shown by the relatively greater size of fraction VIII which evidently consists of unsplit proteins or their very large fragments. The relative content of this fraction, which is 14% in the hydrolyzate of nonspecific γ-globulins, reaches 38% in the antibody hydrolyzate. The lesser splitting of the antibodies is also indicated by the fact that instead of 7 peptides migrating ahead of fraction VIII as in the hydrolyzate of nonspecific γ-globulins, the electrophoretogram of antibody hydrolyzate shows only two large peptides (peptides A and B in Fig. 2). In the antibody hydrolyzate fractions IX and X in most cases are fused together (Fig. 2, a and b), but sometimes they migrate separately (Fig. 2, c).

The enzymatic hydrolyzate of native proetins was also submitted to electrophoresis-chromatography. As a result of this analysis we found only a small quantity of peptides. Since the electrophoresis-chromatographic study of the hydrolyzate of native γ-globulins did not give a great advantage over simple electrophoretic study, it was not used further for study of the native proteins.

Study of the Enzymatic Hydrolyzate of Denatured Nonspecific γ-Globulins and Antibodies

The enzymatic hydrolyzates of denatured γ-globulins and antibodies consists of a considerably greater number of components than the hydrolyzates of the same proteins which have not been first denatured. Electrophoresis-

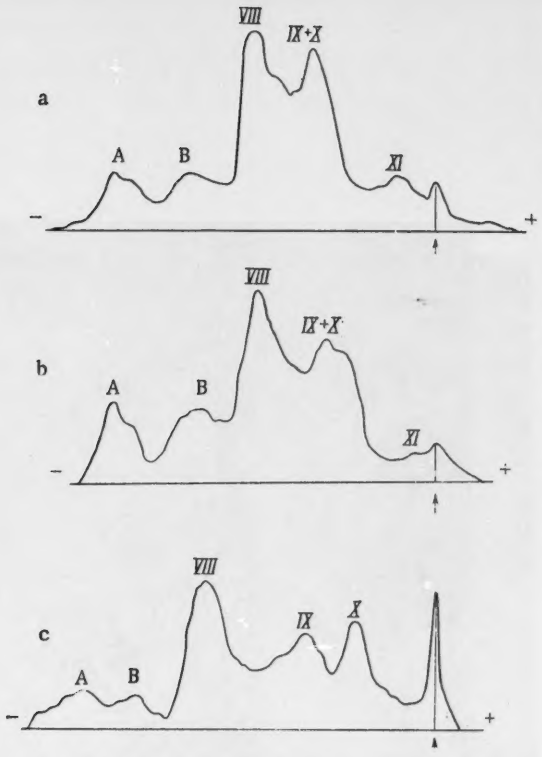

Fig. 2. Electrophoretic separation of enzymatic hydrolyzates of rabbit antibodies. a) Antibody against human serum albumin, isolated from the serum of rabbit No. 56, b) antibody against equine serum albumin, isolated from the serum of rabbit No. 56, c) antibody against human serum albumin, isolated from the serum of rabbit No. 39.

chromatographic analysis of these hydrolyzates permits us to separate a considerably greater number of components than the simple electrophoretic separation.

On the electrophoresis-chromatograms of the enzymatic hydrolyzates of denatured γ-globulins and antibodies there are up to 40 spots. A photograph of one such electrophoresis-chromatogram (enzymatic hydrolyzate of nonspecific γ-globulins) is given in Fig. 3. Most of the spots visible on the electrophoresis-chromatogram are peptides, but we cannot exclude the possibility that individual spots correspond to free amino acids. Under the conditions of our experiments this picture is distinguished by constancy and good reproducibility even in details, although it cannot be asserted that the separation of all the components of the hydrolyzates has led to individual substances.

We get a very similar picture for the electrophoresis-chromatogram of the separation of antibody enzymatic hydrolyzate (Fig. 4). A considerable part of the components isolated from the enzymatic hydrolyzates of the antibodies were identical with the components of the enzymatic hydrolyzate of nonspecific γ-globulins, but close analysis permits us to show small differences between the peptides of the proteins under comparison.

In the electrophoresis-chromatogram of the hydrolyzate from anti-HSA, peptide 31 is practically absent, though it is well shown in the hydrolyzate from nonspecific γ-globulins. In Fig. 4 it is not seen at all, and only traces of it can be seen by looking at the chromatogram in the light. Peptide 6 is also almost absent in the hydrolyzate of anti-HSA though it is also very clearly shown in the hydrolyzate of nonspecific γ-globulins.

The picture seen after separation of the peptides from the hydrolyzate of the second of our studied antibodies, anti-ESA, differs somewhat from the picture seen after separation of the hydrolyzate from the antibody against human

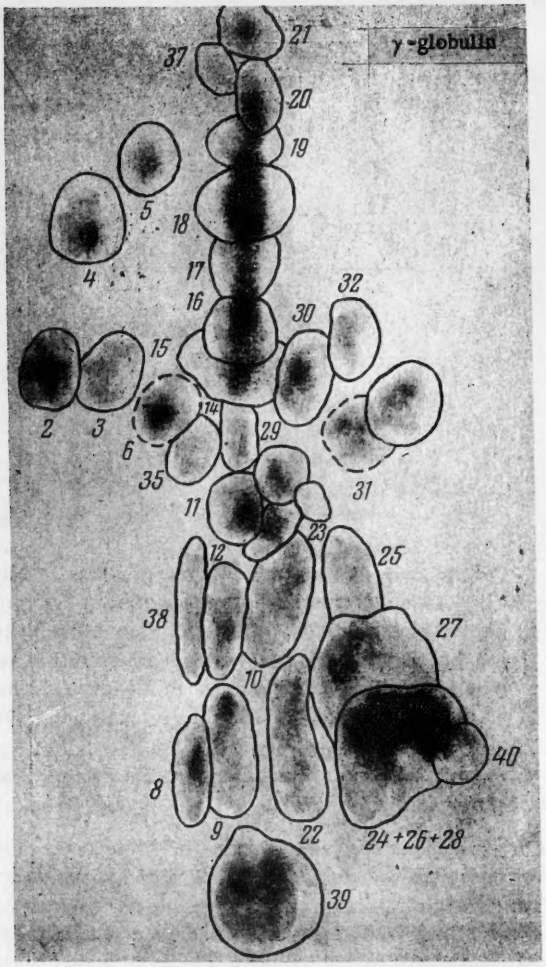

Fig. 3. Electrophoresis-chromatogram of the enzymatic hydrolyzate of denaturated nonspecific γ-globulins of the rabbit. Dotted lines enclose those peptides in which the hydrolyzates of the compared proteins differ from each other.

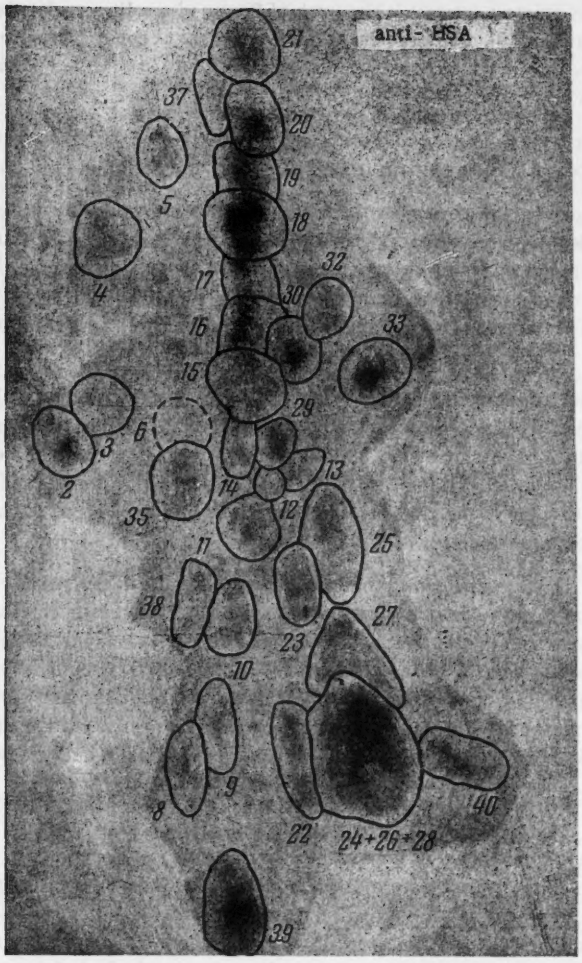

Fig. 4. Electrophoresis-chromatogram of the enzymatic hydrolyzate of denatured antibody against human serum albumin.

serum albumin. Peptide 31 is absent altogether on the electrophoresis-chromatogram of this antibody. The content of peptide 6 in the hydrolyzate from anti-ESA is considerably less than in the hydrolyzate from nonspecific γ-globulins, but all the same, it is more than in the hydrolyzate from anti-HSA.

It is interesting to see that both the antibodies compared in these experiments are isolated from the same rabbit serum, immunized by two proteins.

DISCUSSION

The use of incomplete enzymatic hydrolysis for the study of the antibody structure permits getting a number of interesting results. Of the greatest interest is the work of Porter who split a molecule of rabbit antibody into three fragments with the use of papain [18]. The products formed under these conditions were also studied by Nisonoff and Woernley [19]. An active peptide with sedimentation constant (3.5 S) was formed under the influence of papain by Kul'berg and Tarkhanova [20].

In our study of the antibody submitted to the action of a mixture of trypsin and chymotrypsin, it was split into a considerably greater number of fragments than took place under the action of papain.

Comparison of the peptides obtained under these conditions in enzymatic hydrolysis of two parts of rabbit antibodies and nonspecific γ-globulins showed some differences between these proteins. An especially strong difference was produced by the action of the enzymes on native proteins, not first denatured. In this case the antibodies were split into a smaller number of fragments than was the nonspecific γ-globulin. The antidiphtheria antibody of horse serum was digested to a smaller extent than the nonspecific γ-glubulins by the action of proteolytic enzymes, as Parfent'ev showed [21].

The fraction which we called "nonspecific γ-globulins," if not entirely, then at least to a considerable degree, is a mixture of various antibodies. In connection with this, these results can be interpreted to mean that different antibodies are split into various fragments by the action of proteolytic enzymes, and the splitting of the γ-globulins forms a more heterogeneous mixture of peptides than does the splitting of individual antibodies. It is possible that this difference in ability to be split in the proteins which we compared depends to a considerable extent on primary and also on secondary and tertiary structures.

For an explanation of the differences in primary structures, that is, in the structures of the polypeptide chains of antibodies and nonspecific γ-globulins, the greatest interest lies in the results obtained in the comparison of enzymatic hydrolyzates of proteins which had first been denatured. It was shown that almost all the peptides obtained in the enzymatic splitting of each of the proteins studied in our experiments were similar. This is shown in the similarity of a considerable part of the polypeptide chains in the antibodies and nonspecific γ-globulins studied. It is obviously this which explains the similarity in physicochemical properties of the different antibodies and nonspecific γ-globulins [1, 3, 4] and also the presence of antigenic properties in both of them [22, 23].

However, it should be emphasized that the peptide composition of the hydrolyzates of the proteins compared does not show full identity. The electrophoresis-chromatograms of the hydrolyzates of individual antibodies differ from each other and from the hydrolyzates of nonspecific γ-globulins in the number of peptides and in their relative content. Thus, for example, in the hydrolyzate of anti-ESA, peptide 31 is absent, though it is shown well in the hydrolyzate of the nonspecific γ-globulins. In the hydrolyzate from anti-HSA this peptide is also almost absent. Another difference between the incomplete hydrolyzates of the proteins which were compared is that the relative content of peptide 6 in the hydrolyzate from anti-ESA is much less than in the hydrolyzate from nonspecific γ-globulins. There is still less of this peptide in the hydrolyzate from anti-HSA.

These results favor the view that the polypeptide chains of the separate antibodies and the nonspecific γ-globulins are not completely identical. Evidently they contain portions which differ in alternation of the amino acid chains. Final conclusions as to the presence of such portions can be drawn only after more detailed study of the peptides formed in incomplete enzymatic hydrolyzates.

Our results, which indicate the difference of the peptides formed in the hydrolysis of nonspecific γ-globulins and the antibodies which we studied, as we believe, favor the opinion that these differences between the different antibodies and the nonspecific γ-globulins cannot be due only to differences in form of twisting of the polypeptide chains as first suggested by Pauling [2], an opinion which Horowitz then supported [1]. These results favor the view that under the influence of immunization there is also a change in the first stages of the synthesis of the protein

molecules, that is, a change in the nature of the polypeptide chain which is formed. Such a view was first proposed by Brenel and Horowitz [22] and then developed by a number of authors [25, 26].

The data obtained in the present investigation agrees well with those obtained earlier by us in the study of the relation between the biosynthesis of different antibodies and nonspecific γ-globulins [27, 28].

During the time when our work was already written up there appeared a short report by Gitlin [29] who, like us, used the method of electrophoresis-chromatography for study of the primary structure of rabbit antibodies. His results agree well with ours.

SUMMARY

Under the action of a mixture of proteolytic enzymes (trypsin and chymotrypsin) on native rabbit antibodies (antibodies against horse serum albumin and human serum albumin, isolated from the same serum) and also on nonspecific γ-globulins of rabbit serum, the antibodies were split into a smaller number of peptides (5-6) than the nonspecific γ-globulins, which under these conditions formed 11-13 peptides.

Under the action of the same proteolytic enzymes on the same proteins, first denatured with trichloroacetic acid, they were split into about 40 peptides. A considerable part of these peptides in the enzymatic hydrolyzates of each of the antibodies were identical with the peptides of the hydrolyzates of the other antibody and the nonspecific γ-globulins. However, the peptide composition of the enzymatic hydrolyzate of each of the antibodies studied differed somewhat from the peptide composition of the second antibody and of the nonspecific γ-globulin.

In the enzymatic hydrolyzate of one of the antibodies (the antibody against equine serum albumin) peptide 31 was absent, and in the hydrolyzate of the second antibody (the antibody against human serum albumin) it was found only in traces, although this peptide was well seen in the hydrolyzate from nonspecific γ-globulin. The content of peptide 6 in the antibody hydrolyzates was much less than in the hydrolyzate of the nonspecific γ-globulin.

In conclusion we express thanks to Prof. S. A. Kaplanskii for valuable advice in carrying out this work.

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THE USE OF MICROCIDE (GLUCOSE OXIDASE) FOR DETERMINATION OF BLOOD GLUCOSE IN NORMAL AND DIABETIC SUBJECTS

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In the present paper we report the results of using a domestic preparation of microcide (glucose oxidase) for determination of glucose by an enzymatic method. The enzymatic method has a number of advantages over the chemical method, due to its high specificity, which permits determination of glucose in the presence of other sugars, and also in the presence of different reducing substances of a noncarbohydrate nature.

Glucose oxidase was first discovered by Maksimov in 1904 [1]. In 1928 Müller isolated glucose oxidase from Aspergillus niger and from Penicillium glaucum and established its high substrate specificity [2, 3]. Coulthard and co-workers [4] obtained a highly purified product from Penicillium notatum which they called notatin. In a detailed study of the properties of notatin the authors showed its identity with the glucose oxidase of Müller and the penicillin of Van Bruggen [5]. Glucose oxidase is an enzyme of a flavoprotein nature [4], in which the prosthetic group is an alloxanzine-adenine-dinucleotide [6]. The mechanism of action of glucose oxidase consists in the transfer of two atoms of hydrogen from the six carbon atom of β-D-glucose to oxygen of the air. In this reaction, D-gluconic acid and H_2O_2 are formed.

The use of glucose oxidase for quantitative determination of glucose in biological fluids was first described by Keilin and Hartree [6]. The authors decomposed the H_2O_2 formed in the oxidation of glucose by catalase, and the oxygen which was evolved was calculated using a Barcroft manometer. This method was complex, awkward, and took much time. Later investigators replaced catalase by peroxidase and added to the enzyme mixture an oxygen accepter, o-dianisidine, which became colored on oxidation [7, 8]. Thus the investigators obtained a method for determining glucose colorimetrically. Middleton and Griffiths [9] replaced o-dianisidine by o-tolidine. The method was finally perfected by Marks [10] who used an acetate buffer instead of a phosphate buffer, which stabilized the reagents to a considerable degree. The high specificity and comparative simplicity of the method permitted its use for determination of glucose in blood.

Different authors obtained glucose oxidase from different types of molds: Aspergillus niger, Aspergillus oryzae, Penicillium glaucum, P. resticulosum, and P. notatum [11]. For the determination of glucose we used a preparation of glucose oxidase obtained from the domestic antibiotic "Microcide," purified by the method worked out by Gulyi and Degtyar' [12]. We worked out the conditions for determining glucose with this preparation and made comparative determinations of glucose in blood by chemical and enzymatic methods in normal and diabetic blood.

EXPERIMENTAL PART AND DISCUSSION OF RESULTS

Reagents. For the enzymatic method of determining glucose in blood we used about the same reagents as Middleton and Griffiths [9] and Marks [10]: 0.9% NaCl, 5% $ZnSO_4 \cdot 7H_2O$, 0.3 N NaOH, 1% solution of o-tolidine in absolute alcohol (the o-tolidine was recrystallized from hot absolute alcohol), 0.25 N acetate buffer, pH 4.8 (at this pH of the buffer we found the greatest stabilization of the color in the oxidation of o-tolidine), crystalline peroxidase from horse radish (L. Light and Co., England) 20 mg in 100 ml of acetate buffer; the preparation of glucose oxidase was obtained by the method worked out by Gulyi and Degtyar' [12] from the domestic antibiotic microcide from the culture medium of P. vitale Pidoplitchco et Bilai [13].

The method for obtaining the glucose oxidase was the following: 5 liters of native microcide, whose pH was brought to 6.8 by addition of 40% NaOH, was placed in an adsorber and then treated with 62.5 g of the aluminum

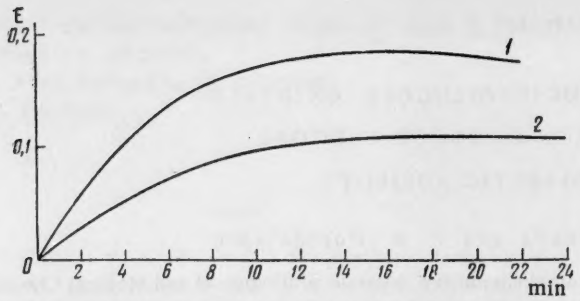

Fig. 1. Change in intensity of color of the reagent after addition of glucose to it. 1) 50 μg glucose, 2) 25 μg glucose.

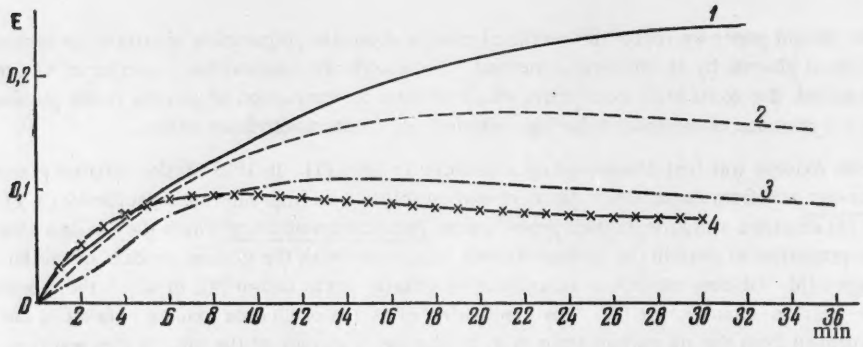

Fig. 2. Effect of pH on results of determination of glucose by the glucose oxidase method. 1) pH 4.4; 2) pH 4.8; 3) pH 5.0; 4) pH 5.4.

oxide four times at intervals of 15 min with constant stirring by a mechanical stirrer (in all, 250 g of Al_2O_3 per 5 liters of microcide). The aluminum oxide with the glucose oxidase adsorbed on it was sucked off on a Buchner funnel and the precipitate was then transferred to a half liter jar with a ground glass stopper. For elution the aluminum oxide was treated with 100 ml of 9% NH_4Cl solution in 0.3 N KH_2PO_4 solution shaken for 20 min on a shaking machine. The eluate was sucked off on a Buchner funnel and the precipitate was twice more eluted on the shaking machine with two portions of 75 ml of 4.5% NH_4Cl in 0.15 N KH_2PO_4 solution.

All the eluates were poured together and cooled to 0°, then precipitated with twice the volume of 96% alcohol cooled to −8 to −10°. The precipitate was centrifuged and dissolved in the minimum amount of ice distilled water. The liquid was separated by centrifuging and the precipitate was again dissolved in the minimum amount of ice water and centrifuged. Extraction of the precipitate was repeated until a colorless centrifugate was obtained. The supernatant liquids were combined and precipitated with a double volume of acetone at −8 to −10°. After centrifuging, the yellow precipitate was successively dissolved and centrifuged in several portions of ice water. The combined, cooled water solutions of the enzyme were again precipitated with a double volume of acetone cooled to −8 to − 10°, centrifuged, and the precipitate dissolved in a small amount of ice water and again centrifuged. The yellow centrifugate was placed over sulfuric acid in a vacuum desiccator. The dried preparation of glucose oxidase was a yellow powder, easily soluble in water. From 5 liters of microcide solution with a bacteriocidal titer of 1 : 16,000 we could obtain from 100 to 200 mg of glucose oxidase preparation.

The working reagent for determination of glucose by the enzymatic method was prepared as follows: to 80 ml of acetate buffer we added 2 mg of glucose oxidase preparation and 5 ml of peroxidase solution. The mixture was stirred, 1 ml of o-tolidine solution was added, and the volume was brought to 100 ml with acetate buffer. The reagent was prepared 1-2 hr before use and could be kept cold in a dark, covered vessel for several days.

Standard glucose solutions, 50, 100, and 150 mg % were prepared in a saturated water solution of benzoic acid (such solutions could be kept in a refrigerator in covered vessels for a long time).

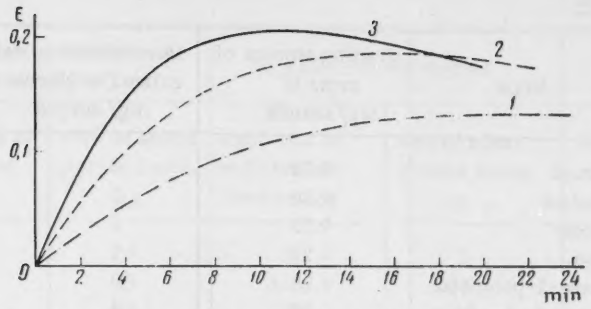

Fig. 3. Effect of different concentrations of glucose oxidase on the course of the enzymatic reaction. 1,2,3) respectively 1,2, and 3 mg % glucose.

Course of the determination. In a centrifuge tube we placed 1.1 ml of NaCl, 0.4 ml of $ZnSO_4 \cdot 7H_2O$, and 0.4 ml of 0.3 N NaOH. To the mixture we added 0.1 ml of blood or 0.1 ml of standard glucose solution, mixed, and centrifuged at 3000-4000 rpm for 5 min. In a determination we took 1 ml of supernatant liquid to which we added 3 ml of working reagent and carefully mixed the contents of the tube. For a control we took 1 ml of water and added 3 ml of working solution. Measurement of the blue color which developed was carried out on a photoelectric colorimeter with a red filter 15 min after addition of the working reagent. Standard curves for determination of the amount of sugar in the blood had to be constructed for each experiment to exclude the small effect of temperature and variation in enzyme concentration. After utilization of the method for construction of the standard curve, one or two points were sufficient. The enzymatic reaction followed Beer's law exactly.

Construction of standard curves could be replaced by calculation of the amount of sugar in the blood by the following formula:

$$S_b = E_b \frac{\Sigma \frac{S_s}{E_s}}{n},$$

where S_b is the amount of sugar in the blood, mg %; S_s the amount of sugar in the standard, mg %; E_b the optical density obtained for blood; E_s the optical density of the standard; n the number of standards taken. In connection with the slight inhibiting action of zinc sulfate and sodium hydroxide on the enzymatic reaction, in order to increase the accuracy of the determination, in the construction of the standard curves we added alkali and zinc sulfates to the glucose in the same proportion and amount as we used in precipitating the blood protein.

The intensity of the color increases constantly (Fig. 1) and reaches its maximum after 13-15 min from the addition of the working reagent to the glucose. Then the intensity of the color remains unchanged for several minutes, and then begins slowly to fall. Therefore, in order to obtain exact results it is necessary to carry out the determination of each sample in exactly 15-17 min after addition of the reagent. The time of building up the maximum color and also the time during which the maximum color is retained is not very stable and depends on a whole series of factors. One of these is the pH of the enzyme mixture.

Study of the effect of pH on the enzymatic reaction showed (Fig. 2) that pH 4.8 is optimum for the work; therefore all the experiments were carried out by us under these conditions. It was established that to maintain the reaction medium a 0.25 N acetate buffer was quite sufficient.

With increasing enzyme concentration the reaction rate increased (Fig. 3), but at the same time there was a decrease of the time in which the maximum color remained unchanged. The best concentration for the enzyme was 2 mg %. At this concentration of the enzyme we could determine from about 20 to 400 mg % of glucose in the blood.

Different preparations of glucose oxidase could have different activities, so that the building up of maximum color could be slow or rapid. Addition or decrease in amount of glucose oxidase permitted a shift in building up the maximum to the desired time.

TABLE 1. Specificity of Action of Glucose Oxidase from Penicillium vitale

Sugar	Starting amount of sugar in mg/sample	Determination, calculated as glucose in mg/sample
Mannose	0.20	0
Galactose	0.20	0
Fructose	0.20	0
Ribose	0.20	0
Glucose-1-phosphate	0.20	0
Glucose-6-phosphate	0.20	0
Maltose	0.20	0
"	5.00	0.015
"	10.00	0.020
Glucose	0.15	0.150
"	0.20	0.200

We also tested the specificity of action of glucose oxidase (Table 1). We showed that it had no catalytic action on mannose, galactose, fructose, ribose, glucose-1-phosphate, glucose-6-phosphate, and maltose taken in amounts of 0.2 mg per sample. The same amount of glucose caused intense coloring of the reagent. Maltose in amounts of 5-10 mg gave a very weak coloration, but the latter could be due to admixtures of small amounts of glucose in the maltose. The data of Table 1 indicates the high specificity of action of glucose oxidase from P.vitale.

Determination of the Content of Glucose in Blood

Parallel determinations of the sugar in the blood from eight donors (mg%) by the method of Hagedorn-Jensen and the enzymatic method gave the following results:

Hagedorn-Jensen method	Enzymatic method	Difference
101	61	40
110	67	44
125	94	31
95	58	37
95	56	39
106	65	41
109	68	41
106	68	38
Average: 106	67	39

Thus, in the determination of glucose by the enzymatic method the average value is 67 mg %* and the variation, from 56 to 94 mg %. In the same donors, the content of sugar in the blood determined by the method of Hagedorn-Jensen varies from 95 to 125 mg %. Hence the average difference between the two methods is about 39 mg % with a variation of 31 to 44 mg %.

In Table 2 we give for comparison the results of determination of glucose in blood by the enzymatic method, method of Hagedorn-Jensen, and the anthrone method [14]. Determination of the sugars by the Hagedorn-Jensen method and with the aid of anthrone gives higher results (respectively 97 and 119 mg %) compared to the glucose oxidase method (81 mg %). This is explained by the fact that the chemical methods determine in addition to glucose

*The average value of glucose in the blood of healthy individuals according to Middleton and Griffiths [9] is 50-90 mg %; according to Marks [10], 80.6 mg %.

TABLE 2. Determination of Different Amounts of Glucose Added to the Blood by the Enzymatic and Chemical Methods

Added glucose, μg	Enzymatic method (glucose oxidase)		Hagedorn-Jensen method		Anthrone method	
	total amount of glucose found, μg	amt. of added glucose found, μg	total amt. of reducing substances, μg	amt. of added glucose found, μg	total amt. of sugars in μg glucose	amt. of added glucose found, μg
0	81	0	97	0	119	0
50	130	49	148	51	168	49
100	180	99	195	98	220	101
150	228	147	249	152	265	146

TABLE 3. Determination of Sugar in the Blood of Diabetics as Measured by Different Methods (mg %)

Patient	Hagedorn-Jensen method	Enzymatic method	Difference
V-va	330	317	13
K-ich	288	259	29
Z-ev	230	200	30
I-on	275	243	32
K-yn	342	308	34
G-an	184	149	35
M-za	319	284	35
G-t	262	221	41
G-ov	187	140	47
L-va	273	223	50
I-ov	275	223	52
T-ov	179	113	66
F-ko	237	168	69
R-ov	344	273	71
R-ich	340	261	79
K-er	277	175	102
G-ov	396	292	104

the different sugars and other reducing substances in the blood. The addition to the blood of different amounts of glucose is equally well determined by all three methods.

There is great value in the use of the glucose oxidase method for diagnosis of diabetes and other diseases related to disturbance of carbohydrate metabolism. During the period of the disease and in the process of treatment it is possible that there is an increase in different reducing substances in the blood which is registered by the Hagedorn-Jensen method as an increase in sugar. This is confirmed by our study of the blood of patients afflicted with diabetes (Table 3). The difference in values for the content of sugar in the blood in diabetes determined by the Hagedorn-Jensen method and with glucose oxidase varies considerably (from 13 to 104 mg %). An explanation of the reason for this variation in diabetes is of undoubted interest and will be the subject of further investigation.

SUMMARY

We have worked out an enzymatic method for determination of glucose using a domestic preparation of glucose oxidase and have carried out comparative determinations of glucose in blood by enzymatic and chemical methods in normal and diabetic subjects.

We express appreciation to the co-workers in the Institute of Epidemiology, Microbiology, and Hygiene (L'vov) for supplying the preparation of microcide.

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DETERMINATION OF THE AMINO ACID COMPOSITION AND N-TERMINAL GROUPS OF CERTAIN PROTEINS IN VIENER AND DONETSKY-650 BARLEY

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It is well known that proteins of barley grain fall into four fractions – albumins and globulins, comprising ~ 30% of the total, grodein, ~ 30%, and glutelin, ~ 40%. Thus, the proteins from this source are either alcohol- or alkali-soluble proteins [1]. Usually biochemical methods have been employed in determining amino acid composition of various fractions of native proteins. However, in 1951 Jensen determined the amino acid composition of a β-globulin which had been purified several-fold by column chromatography [2].

Little effort has been made to identify the terminal groups of barley protein; there is a report of an unsuccessful attempt to determine N-terminal groups of barley grodein using dinitrophenyl derivatives (DNP-derivatives) [3].

It is the purpose of this study to isolate certain protein fractions from barley, and to determine amino acid composition and N-terminal groups of glutelin and grodein by a method used earlier [4]. Initially the proteins were obtained from a standard variety, Viener, which contains a fairly large percentage of nitrogen. Subsequently the study was repeated with proteins from Donetsky-650 barley.

METHODS

Isolation and characterization of protein fractions. Proteins were isolated from Viener barley by the usual method [5], and from Donetsky-650 barley by the method of Folkes and Jemm [6]. The latter method yielded better results. Further purification was carried out by reprecipitation. Albumins were not purified. Glutelins were precipitated from 0.2% NaOH several times. Grodeins were dissolved in warm 70% alcohol, and were then precipitated by cooling; the precipitate was treated with acetone followed by ether, the entire operation, beginning with solution in alcohol, being repeated twice. A viscous mass was obtained which was cut into pieces. All fractions were assayed for nitrogen content by the Kjeldahl method.

Preparation of DNP-derivatives of proteins and their hydrolysis. This was carried out by the usual method [7]. Results of analyses of the various fractions were as follows:

Variety	Protein	Nitrogen content, %	Protein sample, g	DNP-protein sample, g
Viener	Glutelin	16.6	0.18	0.17
"	"	16.7	0.30	0.26
"	Grodein	15.9	0.24	0.23
Donetsky-650	Albumin	11.7	0.30	0.26
"	Grodein	15.3	0.33	0.27
"	Glutelin	16.4	0.31	0.32

The DNP-protein hydrolyzate was freed of humins, concentrated on a water bath to remove excess HCl, and extracted first with ether, then with n-butanol saturated with water [4]. In this manner three fractions were obtained. Each fraction was analyzed by chromatography.

Chromatographic analysis. The following solvent systems were used: 1) phosphate buffer, pH 6.2; 2) borate buffer, pH 2.3; 3) water-saturated phenol; 4) toluene – pyridine – ethylene chlorohydrin – H_2O (1 : 1 : 0.1 : 0.1 by volume); 5) butanol – isoamyl alcohol – ethanol – borate buffer, pH 2.3 (4 : 4 : 1.3 : 6 by volume, upper layer);

6) butanol–CH_3COOH–H_2O (4 : 1 : 5 by volume, upper layer); 7) butanol–CH_3COOH–H_2O (4 : 1.5 : 0.5 by volume); 8) pyridine–CH_3COOH–H_2O (15 : 5 : 3 by volume).

Mixtures were resolved on slow Leningrad paper which had been thoroughly washed with the solvent system prior to use. Where it was desired to treat with ninhydrin to render the spots visible, papers were first washed with 8-oxyquinoline and then with the proper solvent mixture.

RESULTS

Investigation of Ether Extracts of DNP-Protein Hydrolyzates

Ether-soluble DNP-amino acids were analyzed in five different solvent systems (1-5).

Chromatographic analysis of the ether fraction of a DNP-glutelin hydrolyzate from Viener barley. The hydrolyzate was applied with a pipette to a 40 × 32 cm paper. It was applied in a line 24 cm in length and 0.3-0.5 cm in width. DNP-amino acid markers were applied in spots with a capillary. After development, the chromatogram was cut lengthwise into strips 2 or 3 cm in width, and the yellow DNP-amino acid strips were also cut out. R_f's were estimated from the point of intersection of the midline of the strip and the line of application.

When chromatograms were developed with sodium phosphate solution, the DNP-glutelin hydrolyzate was resolved into three spots (Table). The first spot (R_f 0.3-0.4) corresponded to dinitroaniline (DNA, R_f 0.3-0.4), the second (R_f 0.7-0.8) to dinitrophenol (DNP, R_f 0.83-0.85), and the third (R_f 0.8-0.9) appeared to correspond to DNP-glutamic or DNP-aspartic acid, these being unresolved in aqueous solution.

R_f Values of Components of the Ether-Soluble Fraction of DNP-Glutelin Hydrolyzate

Solvent–phosphate buffer

Expt. No.	DNP-glutelin hydrolyzate			DNP	DNP-aspartic acid		Dinitroaniline
	spot 1	spot 2	spot 3		spot 1	spot 2	
1		0,78	0,935	0,83	0,82	0,94	~0,32
		0,77	0,950				
	0,38	0,79	0,935				
	0,37	0,795	0,925				
	0,35	0,78	0,955				
		0,86	0,945				
		0,81	0,930				
2			0,92	0,85	0,84	0,945	
		0,84	0,94				
		0,87	0,95				
		0,77	0,93				
		0,89	0,95				
		0,89	0,96				

When chromatograms were developed in water-saturated phenol or solvent 4, the same results were obtained.

Further analyses of the ether fraction of a DNP-glutelin hydrolyzate from Viener barley. In these solvent systems, DNP-glutelin hydrolyzate is separated into three spots. Two of these migrated in a fashion parallel to DNP and DNA. The third spot (R_f 0.8-0.9 in aqueous solution; 0.45-0.5 in phenol; 0.06–0.1 in solvent 4) appears to be identified with DNP-aspartic or DNP-glutamic acid. This is confirmed by the following check experiment:

The spot in question was extracted with 0.1% $NaHCO_3$, the extract being concentrated by evaporation on a water bath and once again chromatographed in the same solvents. With this procedure, the same results were obtained.

In order to determine the N-terminal amino acid of glutelin, it would be necessary to separate DNP-aspartic and DNP-glutamic acid. Accordingly, a mixture of alcohols saturated with buffer [8] at an acid pH, 2, was tested (solvent 5). The DNP-amino acids were separated by repeated treatment of the papers with this solvent system. The chromatogram shown in Fig. 1 shows that with three passages of solvent complete separation of DNP-aspartic and DNP-glutamic acid occurs, with the latter migrating at a faster rate.

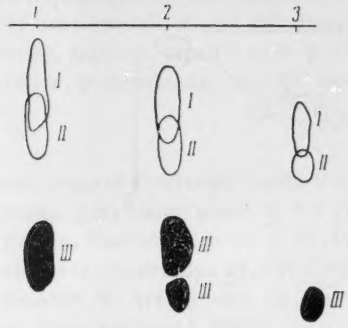


Fig. 1. Chromatogram of solutions of DNP-aspartic and DNP-glutamic acids. Solvent 5 (see "Methods"). DNP-derivatives: 1) aspartic acid; 2) aspartic and glutamic acids; 3) glutamic acid. I, II, III indicate number of passages of solvent.

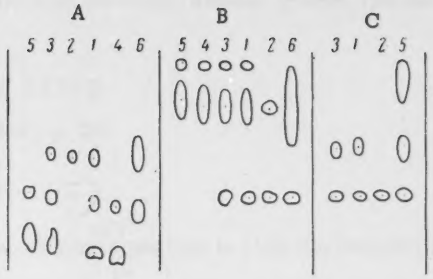


Fig. 2. Chromatogram of the ether-soluble fraction of DNP-protein hydrolyzates from Donetsky-650 barley. A) Solvent 2; B) solvent 4; C) solvent 5. 1) DNP-glutelin; 2) DNP-grodein; 3) DNP-albumin; 4) DNP-glutamic acid; 5) DNP-glutamic + DNP-aspartic acids; 6) DNAn or DNAn + DNP.

When DNP-glutelin hydrolyzate is chromatographed in solvent 5, two spots are obtained, of which the upper has an R_f greater than that of DNP-aspartic acid and similar to that of DNP-glutamic acid. The second spot is parasitic.* It may therefore be considered that the N-terminal amino acid of glutelin from Viener barley is glutamic acid.

Analysis of the ether-soluble fraction of a DNP-grodein hydrolyzate from Viener barley. In extraction of this hydrolyzates the ether layer became very weakly colored. The hydrolyzate was resolved by chromatography into two components corresponding to DNP and DNAn. The attempt to determine N-terminal groups in grodein by the use of dinitrophenyl derivatives was therefore unsuccessful.

Analysis of the ether-soluble fraction of hydrolyzates of DNP-albumin, DNP-grodein, and DNP-glutelin from Donetsky-650 barley. Hydrolyzates were chromatographed in the same solvent systems as used in the previous experiments. Fig. 2 shows that albumin and glutelin in Donetsky-650 barley have as their N-terminal group glutamic acid, while grodein contains no N-terminal group.

Investigation of the Butanol Extract of a DNP-Glutelin Hydrolyzate

After ether extraction, the DNP-glutelin hydrolyzate was treated with water-saturated butanol. All water-soluble DNP-amino acids, and also some of the amino acids formed during protein hydrolysis, go into the butanol.

R_f values of water-soluble DNP-amino acids separated in a butanol–CH_3COOH–H_2O mixture were:

DNP-glutelin hydrolyzate			DNP	Mono-DNP-lysine	DNAn
spot 1	spot 2	spot 3			
0.46	0.67	0.88	0.86	0.64	0.9
0.43	0.63	0.87	0.85	0.54	0.9

Judging by these figures, spot 1 is probably N-DNP-histidine, since it is weakly colored and when sprayed with ninhydrin becomes brown. Spot 2 migrates in parallel fashion with mono-DNP-lysine, and spot 3 is evidently a mixture of DNP and DNAn. Similar results were obtained with chromatographic analysis of a DNP-glutelin hydrolyzate from Donetsky-650 barley.

Investigation of the Water-Soluble Fraction on DNP-Glutelin and DNP-Grodein Hydrolyzates

Sometimes DNP-protein hydrolyzates remained light yellow in color even after extraction with ether and butanol. This however, did not interfere with chromatographic analysis.

* A more appropriate translation would be "The second spot corresponds to DNP or DNAn or both."—Publishers Note.

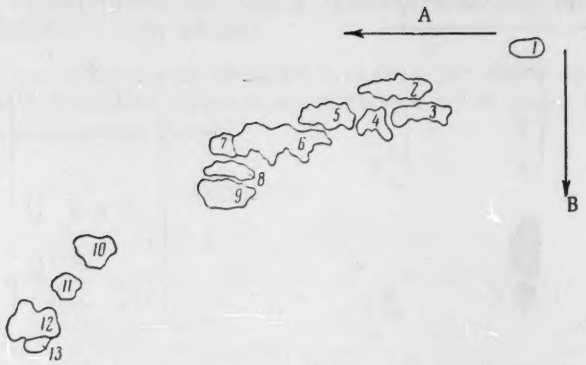
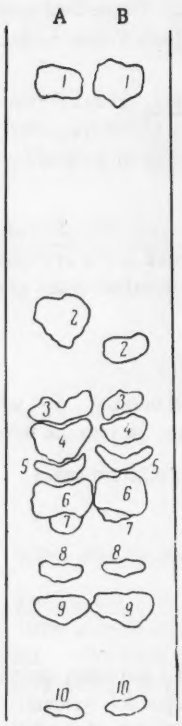


Fig. 3. Two-dimensional chromatogram of the water-soluble fraction of DNP-glutelin hydrolyzate. Solvents: 8 (direction A) and 6 (direction B). 1) Cystine; 2) arginine; 3) aspartic acid; 4) glycine; 5) serine; 6) glutamic acid; 7) threonine; 8) alanine; 9) proline; 10) tyrosine; 11) valine + methionine; 12) phenylalanine; 13) leucine



As the two-dimensional chromatogram of Fig. 3 shows, the water-soluble fraction of DNP-glutelin hydrolyzate contains 14 amino acids. Lysine and histidine, which were found in the butanol extract as their mono-DNP-derivatives, are not present.

In order to compare the amino acid composition of glutelin and grodein, one-dimensional chromatograms were prepared which could be used for quantitative determination of amino acids. To a sheet of slow chromatographic paper 50-60 cm long were applied in fine lines DNP-glutelin hydrolyzate, DNP-grodein hydrolyzate, and a mixture of amino acids and tropeolin; the latter migrates at the same rate in this solvent system as does the fastest amino acid—leucine. The lower edge of the paper was cut in notches in order to prevent the solvent from piling up at the paper's edge and to encourage it to flow off freely. Solvents 6 and 7 were used. The chromatogram was placed in the chamber and solvent was allowed to flow through $^1/_3$ its length the first passage, $^1/_2$ its length the second, and $^3/_4$ its length the third. After each passage the chromatogram was removed and dried. After each each passage the chromatogram was removed and dried. After the third passage, two more passages were allowed, the solvent being permitted to flow off the end of the paper until the red tropeolin spot had reached the tip of a tooth. Fig. 4 shows that in this manner it was possible to separate almost 14 amino acids.

Fig. 4. Upper portion of a chromatogram of the aqueous layer remaining after extraction of DNP-glutelin and DNP-grodein hydrolyzates from Viener barley with ether and butanol. Solvent 7. A) DNP-glutelin hydrolyzate; B) DNP-grodein hydrolyzate. 1) Cystine; 2) arginine; 3) serine; 4) aspartic acid; 5) glycine; 6) glutamic acid; 7) threonine; 8) alanine; 9) proline; 10) tyrosine.

Similar results were obtained with chromatographic analysis of aqueous solutions of DNP-protein hydrolyzates from Donetsky-650 barley.

SUMMARY

The N-terminal group of glutelin from Viener and Donetsky-650 barleys is glutamic acid. In grodein no N-terminal groups were found using the method of dinitrophenyl derivatives.

Amino acid composition of glutelin and grodein from Viener and Donetsky-650 barleys was qualitatively determined by one-dimensional and two-dimensional paper partition chromatography. Sixteen amino acids were found: cystine, arginine, aspartic acid, glycine, serine, glutamic acid, threonine, alanine, proline, tyrosine, valine and methionine, phenylalanine, leucine, histidine and lysine.

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FREE AMINO ACIDS IN E. coli AFTER BLOCKADE OF PROTEIN SYNTHESIS WITH CHLOROTETRACYCLINE

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The problem of the formation and accumulation of separate amino acids in the protein synthesis of microorganisms from ammonium salts and glucose has remained obscure up to the present time, in spite of the large number of investigations of this subject.

In 1947 Taylor [1] noted that microorganisms did not form large internal stores of free amino acids. Using different modern methods of investigation, Dagley and Jonson [2] found a large number of amino acids in mocrobes, but they did not succeed in finding several amino acids which compose the bacterial protein. In our investigations, we too, could not find many of the amino acids in the collection of free amino acids of E. coli and proposed that the reason for this was the rapid uptake of these amino acids into the protein during its synthesis.

In order to verify such a hypothesis we decided to investigate the accumulation of amino acids in microbial cells under conditions in which the protein synthesis was inhibited, while the synthesis of amino acids could proceed [3]. To accomplish this we carried out experiments under anaerobic conditions which as in animal tissues decreased the synthesis of E. coli proteins, and could not affect the synthesis of amino acids; or we added 2% propyl alcohol which inhibited the synthesis of protein, but did not affect the synthesis of aspartic acid from fumarate and ammonia in microorganisms. In these experiments we did not succeed in observing an accumulation of amino nitrogen from the keto acids and ammonia in E. coli Communis and E. coli Communior either under anaerobic conditions or in experiments in which propyl alcohol was used.

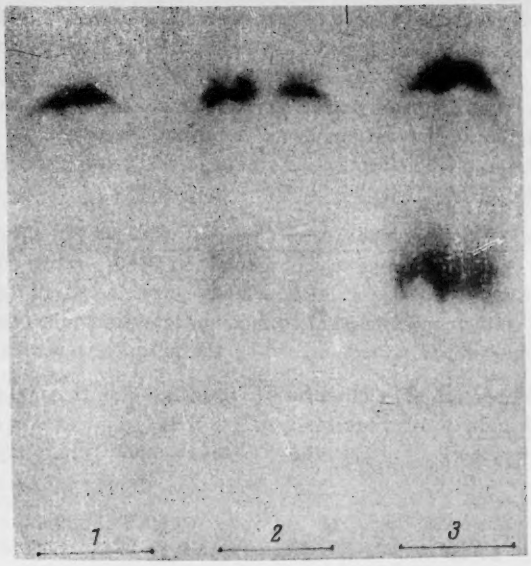
Lately Katanuma [4] has shown the presence of enzymes in microorganisms which activate ammonia for the synthesis of amino acids. The activation of ammonia, according to Katanuma, takes place with the formation of adenylamidate (AMP ~ NH_2) from NH_3 and adenosinetriphosphate. Since the formation of the latter during anaerobiosis must be decreased, then, evidently, in our previous experiments it could have been the formation of the amino acids themselves which were disrupted. It is possible that propyl alcohol, while not interfering with the specific synthesis of aspartic acid, inhibited the formation of other amino acids. Consequently we decided to reinvestigate this problem, using several antibiotics as specific factors which would inhibit protein synthesis.

According to the data of Gale and Folkes [5], chloramphenicol and tetracycline, and particularly chlortetracycline are capable even at low concentrations of inhibiting the protein synthesis in microorganisms from the appropriate amino acids. According to Loomis' data [6], oxidative phosphorylation is inhibited by these antibiotics only when they are used at high concentrations, and consequently, in low concentrations it cannot interfere with the activation of ammonia, occurring with the mediation of ATP.

METHODS

The experiments were carried out with E. coli culture 675.* Flasks with solid nutritive broth** were inoculated with an 18 hr culture. After 18 hr the microbial growth was washed off with physiological solution, separated by centrifugation and washed again with physiological saline. From the washed cells a suspension of the necessary density was prepared. The number of bacteria in a 1 ml suspension was established by standard turbidity. The flasks contained

*The strain was obtained from the Culture Museum of the L. A. Tarasevich Vaccine and Serum Institute, Moscow.
**The solid nitritive agar is a product of the N. F. Gamalea Institute of Epidemiology and Microbiology, Moscow.



Chromatograms of free amino acids of E. coli, incubated in the absence and in the presence of chlortetracycline. Composition of the sample for the first two experiments: Suspension of E. coli, buffer, glucose, NH_4Cl; for the third, the same + 50 μg/ml of chlortetracycline. 1) incubation was not carried out; 2 and 3) incubation for 3 hr.

1/15 M phosphate buffer, pH 7.5, and other ingredients in the following final concentrations: glucose 0.05 M, NH_4Cl 0.02 M, α-ketoglutaric, glutamic, and other amino acids 6 10 M.*

The total volume of the incubated sample was 8 ml. The samples were placed in small flasks and gassed with a mixture consisting of 95% O_2 and 5% CO_2. The flasks were incubated in a stirred water bath at 37°. At the end of the incubation 7 ml of each sample was withdrawn into a centrifuge tube, 1 ml of 80% TCA was added, and the mixture was placed overnight in a refrigerator; it was then centrifuged. The original unincubated samples were treated in the same manner, immediately after the addition of the suspension of microbes to the sample.

Protein nitrogen was determined by the Biuret reaction [7]. For this purpose, the protein precipitates were washed twice with cold 5% TCA, 3 ml of 1 N KOH was added, and the mixture was placed in a thermostat at 37° for 18 hr. To the alkaline solution of protein, 1 ml Biuret reagent was added, prepared according to Khesin's instructions [8], and read colorimetrically on a photoelectric colorimeter with a green filter. Since the density of the alkaline solutions of protein varied greatly, the color was read against the same alkaline solutions of protein to which Biuret reagent had not been added. A standard curve was used for calculations, which had been constructed from known quantities of bacterial protein, the nitrogen of which after mineralization was determined by isothermic distillation.

Ammonia nitrogen in the TCA-extracts was determined in Conway cups. In order to displace the NH_3, 0.5 N NaOH was added with bromothymol blue as an indicator. After the appearance of a blue color, an excess of alkali was added to the amount of 0.5 ml per 1 ml sample. After the determination of ammonia by titration, the contents of the outer chamber were used for the determination of amino nitrogen according to the method of Cocking and coauthors [9]. It was previously established that there was complete agreement between the content of amino nitrogen, obtained by the method described above for the distillation of NH_3 and the vacuum distillation of NH_3, recommended by Cocking. To determine the amino acids chromatographically, 8 ml of centrifugate was extracted 5 times

*Since the chlortetracycline forms a firm complex with alkaline earth metals and could be "neutralized" by them, we did not add any other salts to the samples.

with ether in order to remove the TCA; the mixture was made alkaline with 0.5 N NaOH to bromothymol blue, and evaporated to dryness in a vacuum by heating to 40°. The residue was dissolved in water, neutralized, and again evaporated to dryness. The dry residue was extracted with 0.5 ml of ethanol, cooled to 0°, filtered, and the alcohol extracts placed in bands on chromatographic paper. The amino acids were separated in a mixture of n-butanol-acetic acid-water (4 : 4 : 1); the solvent was circulated 2-3 times. In some cases two-dimensional chromatography with 80% phenol as a second, was used to identify the amino acids. The chromatograms were developed with 0.5% ninhydrin in acetone at 65° for 30 min.

Accumulation of amino acids after blockade of protein synthesis in E. coli with chlortetracycline. According to our previous experiments, chlortetracycline in a concentration of 50 μg/ml, completely inhibits the synthesis of protein even in the most viscous suspensions which we used. Investigation of the effect of chlortetracycline in the indicated concentrations on the consumption of ammonium nitrogen and the formation of amino nitrogen in E. coli, cultivated on a glucose-ammonia medium, showed the following (average data after 3 hr incubation):

Suspension of E. coli, × 10^9 per ml	Chlortetracycline, μg/ml	Decrease in N-NH_3, mg/sample	Increase in N-NH_2, mg/sample
2-3	–	0.36	0.04
	50	0.09	0.04
20-30	–	0.72	0.03
	50	0.14	0.08

The data shows that in the absence of chlortetracycline amino nitrogen was accumulated, both in the dilute and in the thick bacterial suspensions, in an amount equal to $^1/_{10}$ to $^1/_{20}$ the amount of absorbed ammonia nitrogen. In the presence of chlortetracycline the requirement for ammonia nitrogen in the dilute and dense suspensions was 4-5 times less. However, a marked increase in amino nitrogen accumulation was observed in dense suspensions when protein synthesis was blocked. In these samples the amount of amino nitrogen was approximately $^1/_2$ the ammonia nitrogen consumed.

Qualitative and quantitative composition of free acids in E. coli, strain 675. It was of interest to determine which amino acids were accumulated in E. coli cultivated in a glucose-ammonia medium in the absence and presence of chlortetracycline.

As may be seen from the chromatograms of free amino acids of E. coli (figure) the position of the spots was approximately the same in all three cases. At the same time, almost all spots of the samples from the microorganisms incubated in the presence of chlortetracycline (sample 3) were more intensively stained. In all three cases the two upper spots were found to be the most distinct; these spots were those of valine and leucine, the lower spot corresponding to glutamic acid. The other amino acids, apparently, were present in relatively small amounts. In tests to identify these amino acids, we found traces of cystine and α- or β-alanine. However, we could not obtain clear evidence of the accumulation of these amino acids in very small quantities.

Gershenfeld and Bernstein [10] found only leucine in a free state in E. coli cells. Dagley and Jonson [2] found a considerably larger number of amino acids in microorganisms in different periods of growth both in the free state and bound in peptides. According to their data, glutamic acid was the first to appear in filtrates, followed by aspartic acid, serine, glycine and alanine; finally, valine, methionine, leucine and isoleucine.

Sorm and co-workers [11] cultivating E. coli on a glucose-mineral medium in the presence of chloramphenicol found only alanine, valine and leucine in the medium. Cultivating coliform bacterias with oxytetracycline, Snell and co-workers [12] found a considerable accumulation of D-glutamic acid. The distinct differences in the amounts of accumulated free amino acids are very noticeable. Probably this is a result of the different rates of further transformation or formation.

Rate of deamination of some amino acids in suspensions of E. coli and the effect of chlortetracycline on this process. Comparatively recently Otsuka and co-workers [13] have shown that under strongly aerobic conditions, favorable for the growth of microorganisms, glutamic acid is not accumulated, while in anaerobic conditions with slow growth the amount of glutamic acid is increased in a 10-day period. On this basis the authors proposed a definite regime of aeration for the fermentative production of glutamic acid by means of microorganisms. These

Deamination of Amino Acids in Suspensions of E. coli, Strain 675, and the Effect of Chlortetracycline on Deamination (Suspension of E. coli, × 10^9/ ml; time of incubation 2 hr. The average of three experiments is given.)

No. of sample	Added: amino acids	Added: chlortetracycline μg/ml	Formation of N-NH_3, mg per sample	Decrease in N-NH_2, mg per sample
1	L-glutamic acid		0,52	0,55
	The same	50	0,50	0,45
2	" "	400	0,15	0,13
3	DL-glutamic acid	50	0,30	0,33
4	L-aspartic acid		0,55	0,49
		50	0,60	0,49
5	α-DL-alanine		0,60	0,55
	The same	50	0,60	0,55
6	Glycine		0,28	0,20
	The same	50	0,24	0,16
7	L-cystine	50	0,15	0,13
8	β-alanine	50	0,05	0,06
9	DL-valine		0,10	0,02
	The same	50	0,04	0
10	DL-leucine		0,11	0
	The same	50	0,08	0,06

data, as well as the data of Dagley and co-workers [14] showing that the amount of free amino acids in microorganisms is always less in aerated cultures than in non-aerated, impelled us to assume that the different rate of deamination of individual amino acids can, in some degree, be explained by their differing accumulation in the free state when protein synthesis is blocked. Originating from this assumption, we investigated in the following series of experiments the rate of deamination of those amino acids which either in data from the literature or according to our data occurred in the largest quantities in the free state, and compared it with the rate of deamination of other amino acids which were found in considerably smaller amounts. In these experiments we also investigated whether chlortetracycline had an effect on the deamination of these amino acids (Table).

As may be seen from these data, valine, leucine, β-alanine and cystine practically did not deaminate under these conditions, while all other amino acids which we tested were deaminated to a greater or lesser degree. Comparison of the rate of deamination of L-glutamic acid and its racemate showed that D-glutamic acid was deaminated very slightly [15]. Chlortetracycline in amounts of 50 μg/ml had no marked effect on the process of deamination in a single case, while at higher concentrations its inhibitory action on the deamination of L-glutamic acid was obvious (Table, sample 2). Thus, our experiments demonstrated a certain correlation between the rate of deamination of individual amino acids and the intensity of their accumulation.

There were some discrepancies between our observations and the data of Sorm and co-workers [11]; they found an accumulation of alanine, which according to our data was deaminated very quickly. However, it is possible that the strain of E. coli used in Sorm's work deaminated alanine more slowly or that under the conditions of Sorm's experiments, deaminated β-alanine was accumulated slowly; its position on chromatograms is almost the same as that of α-alanine.

SUMMARY

When the protein synthesis of E. coli is inhibited by chlortetracycline at a concentration of 50 μg/ml, the accumulation of free amino nitrogen occurs somewhat more slowly than in the absence of chlortetracycline.

Valine, leucine and glutamic acid were found in the greatest amounts after paper chromatography. Investigation of the rate of deamination of several amino acids in suspension of E. coli showed that valine, leucine, D-glutamic acid, cystine and β-alanine are deaminated very slowly, while L-glutamic acid, α-alanine and L-aspartic acid are deaminated quickly.

It is suggested that the composition of the free amino acids accumulating in microorganisms depends on the rate of deamination of the individual amino acids.

We wish to extend out deep gratitude to Prof. S. Ya. Kaplanskii for consultation.

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THE EFFECT OF IONIZING RADIATION ON ACETYLCHOLINE METABOLISM IN MACACA-RHESUS MONKEYS*

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In previous investigations [1] one of us studied the effect of roentgen irradiation on total cholinesterase (TCE) activity of some tissues and blood serum of macaca-rhesus monkeys.

Since in those experiments we studied the TCE activity only, characterizing a single phase of acetylcholine metabolism, in this study we simultaneously investigated changes in the cholinesterase activity as well as acetylcholine content (AC) in tissues of irradiated monkeys. In addition to TCE activity and total AC content, we determined the specific acetylcholinesterase (ACE) activity and nonspecific cholinesterase (NCE) activity as well as free (conditionally) and bound AC in the same tissues of the experimental animals.

METHODS

Two series of experiments were performed. In the first series 4 monkeys weighing 2.2-2.3 kg were subjected to γ-irradiation with Co^{60} at a dose of 600 r and a dose energy of 93.7 r/min. Of this first group of monkeys, two were killed on the first day and two on the third day after irradiation. The second series of experiments were carried out on monkeys weighing 4.5-10.8 kg, put to death 2-3 years after roentgen irradiation at a dose of 300-700 r.

Three monkeys weighing 2.0-5.8 kg (kept in Sukhumi) and 5 monkeys weighing 2.1-7.5 kg (kept in Moscow** conditions) served as controls.

For the tissue investigations, the animals were decapitated; the brain, liver and small intestinal mucosa were quickly removed The material removed was placed in ice or in liquid nitrogen. Cholinesterase activity was determined by the method of Zubkova and Pravdich-Neminskaya [2], using as substrates for TCE, ACE and NCE, respectively, preparations of AC, acetyl-β-methylcholine and benzoylcholine.

The AC content was determined biologically on the dorsal muscle of the leech. The investigated tissue samples were homogenized and extracted with Ringer's solution, pH 7.6 (1:10). To determine free AC the extraction was carried out in ice; in the case of total AC the extract was heated in a boiling water bath for 5 min (in order to denature the bound AC-proteins). The extract was introduced into a vessel in which a preparation of leech dorsal muscle was placed, and the magnitude of the muscle contraction was chemographically measured, comparing it afterwards with the magnitude of the contraction caused by a solution of AC of known concentration. The content of bound AC was determined by the difference between the amounts of total and free AC.

RESULTS OF THE INVESTIGATION

Data on the content of free and bound AC are presented in Table 1. In the tissues we investigated free and bound AC content was lowest in liver, highest in small intestinal mucosa; AC of the brain occupied an intermediate position. Further, the amount of free AC was higher than bound in intestinal mucosa, while in brain and in liver the free AC content was not significantly different from the bound. One to three days after irradiation of the animals, an increase in concentration both of the free (2.5-7 times) and bound (2 times) AC was noted in the intestinal

*The present study, as was the previous study, was carried out at the base of the Institute of Experimental Pathology and Therapy of the Academy of Medical Sciences of the USSR in Sukhumi, the management of which kindly allowed us this opportunity.

**These animals were kindly submitted by the Institute for the Study of Poliomyelitis, Academy of Medical Sciences of the USSR, Moscow.

TABLE 1. Content of Free and Bound Acetylcholine (in μg %) in Small Intestinal Mucosa, Brain and Liver of Macaca-Rhesus Monkeys with Acute Radiation Disease and its Long Term Effects

Small intestine		Brain		Liver	
I	II	I	II	I	II
Normal					
600	200	150	50	30	10
500	200	100	50	20	10
600	200	200	50	40	10
270	390	120	200	30	60
390	330	40	140	20	70
550	440	210	70	40	70
210	110	80	20	10	10
140	150	50	40	10	-
First day after irradiation					
3500	500	—	—	30	20
2000	500	—	—	20	80
Third day after irradiation					
1000	500	—	—	40	20
1000	500	—	—	40	20
Longterm effects					
1800	200	400	100	40	10
800	200	300	100	70	10
1000	500	200	100	40	10
1000	800	800	200	400	100
1800	200	700	100	500	100

Note: I, free; II, bound acetylcholine.

mucosa, while in the liver no statistically verifiable changes in the concentration of free and bound AC occurred. To our regret, for technical reasons we were not successful in carrying out the determination of AC content in the brain of the irradiated animals.

In monkeys tested 2-3 years after irradiation, the amount of bound AC in all the tissues did not differ markedly from the normal. At the same time the concentration of free AC in these animals in the intestinal mucosa, brain and liver was significantly higher than normal.

In Table 2 data are presented on the changes on TCE, ACE and NCE activity in tissues of liver, brain, and intestinal mucosa of macaca-rhesus monkeys after the action of ionizing radiation. The values for OCE, ACE, and NCE activity in the control animals in the tested tissues can be arranged in the following order: brain > intestinal mucosa > liver.

A day after γ-irradiation of the animals a significant increase in NCE activity was observed in the intestinal mucosa, together with a tendency to increased ACE activity; this, naturally, was the reason for the increase in TCE activity. In the liver during this period, statistically verifiable changes in TCE, ACE, and NCE activity were not found. Three days after the action of ionizing radiation a further increase in NCE activity was found, as well as some statistically insignificant increase in ACE activity in intestinal mucosa. Consequently, the TCE activity in this tissue continued to increase. An increase in NCE activity but no change in ACE activity was observed in liver tissue in these periods. Determinations of TCE, ACE and NCE activity in brain tissue of irradiated animals in this series of experiments were not successful.

In animals irradiated 2-3 years before the investigation, TCE, ACE, and NCE activity were increased in liver tissue. TCE and ACE activity were also increased in small intestinal mucosa for these same animals, and NCE activity had a tendency to increase. In brain tissue, on the contrary, ACE activity was lower than normal, while NCE activity did not change; TCE activity was thereby decreased.

TABLE 2. Total Cholinesterase, Acetylcholinesterase and Nonspecific Cholinesterase Activity in Liver, Brain and Small Intestinal Mucosa of Macaca-Rhesus Monkeys with Acute Radiation Disease and its Long-Term Effects (in ml Acetylcholine decomposed per 1 hr)

TCE			ACE			NCE		
intestine	brain	liver	intestine	brain	liver	intestine	brain	liver
Normal								
0,64	1,00	0,47	0,21	0,51	0,10	0,47	0,55	0,26
0,74	1,07	0,51	0,22	0,52	0,12	0,52	0,57	0,28
0,67	1,05	0,48	0,21	0,53	0,13	0,49	0,58	0,24
0,62	1,03	0,42	0,29	0,48	0,22	0,28	0,58	0,21
0,71	1,98	0,47	0,39	0,47	0,23	0,31	0,47	0,21
0,73	1,06	0,51	0,36	0,55	0,27	0,28	0,50	0,24
First day after irradiation								
0,89	—	0,47	0,42	—	0,15	0,61	—	0,26
0,93	—	0,49	0,30	—	0,17	0,76	—	0,27
Third day after irradiation								
1,03	—	0,57	0,35	—	0,17	0,76	—	0,36
1,09	—	0,65	0,43	—	0,22	0,86	—	0,42
Long-term effects								
0,79	0,88	0,69	0,42	0,47	0,39	0,45	0,49	0,40
0,88	1,02	0,80	0,49	0,42	0,42	0,48	0,58	0,44
0,89	0,94	0,82	0,45	0,48	0,44	0,47	0,53	0,45
0,80	0,92	0,73	0,44	0,49	0,40	0,45	0,52	0,42
0,86	0,96	0,79	0,47	0,51	0,44	0,49	0,53	0,45

The increased content of free and bound AC which we found in monkey mucosa 1-3 days after lethal γ-irradiation, in spite of the simultaneous increase in TCE activity because of increased NCE and ACE activity, particularly the latter, was obviously evidence of the pronounced increase in AC synthesis in this tissue under the existing conditions. Analogous changes occurred later in the liver (3 days after γ-irradiation) and were less clear cut.

The increased synthesis of AC also took place, apparently, in liver tissue and small intestinal mucosa in animals tested 2-3 years after irradiation. A considerable increase in the concentration of free AC with increase in TCE activity (because of ACE and particularly NCE activity) were also found in these tissues. We found an increased content of free AC (4 times that of the normal) in the brain of these same animals; this could be completely explained according to our opinion, by some decrease in TCE activity (due to a fall in ACE activity). Here, as in the intestine and liver, there was probably an increased synthesis of AC.

By comparing the values of free and bound AC concentrations in tissues of the irradiated animals, a more significant change in the amount of free AC could be noted than in the bound. Thus, the content of free AC sharply increased in 1-3 days and was high 2-3 years after the action of ionizing radiation in intestinal mucosa, in liver, and in brain. However, increase in bound AC was found only in intestinal mucosa 1-3 days after irradiation.

It is interesting to compare data on the changes of TCE, ACE and NCE activity in tissues of monkeys killed 1-3 days and 2-3 years after irradiation. In the first case the TCE activity in the small intestinal mucosa and liver is the result of increase in the NCE activity. With a long time lapse after irradiation injury TCE in the intestinal mucosa increased, but decreased in the brain, mainly because of the corresponding increase and decrease in the ACE activity; in the liver, however, in this case, increase in the TCE activity is a consequence of the concomitant increase in ACE and NCE activity.

It must be pointed out that French and Wall [3], investigating the NCE activity in monkey small intestine after roentgen irradiation at a dose of 800 r, did not find changes in activity either at 2 or at 7-8 days after irradiation. It is possible that this difference from our data can be explained by different experimental conditions.

The significant shifts in the metabolism of AC, found in the monkey even some years after radiation injury is evidence for the serious disruption of AC conversion by ionizing radiation. It must be noted that radiation shifts

in the metabolism of AC in monkeys significantly differ in detail from similar changes in other experimental animals, rats, for example [4].

SUMMARY

In macaca-rhesus monkeys the normal content of free acetylcholine in the mucosa of the small intestine was higher, as it was in brain and liver, than bound acetyl choline.

The total cholinesterase activity and, particularly, the activity of acetylcholinesterase and non-specific cholinesterase in control monkeys is highest in brain, followed by intestinal mucosa and liver.

One to three days after γ-irradiation of the monkey at a dose of 600 r the amount of free and bound acetylcholine in the mucosa of the small intestine increased, while it decreased in liver. The total cholinesterase activity in the mucosa of the small intestine during this period increased, in general because of the increase in the activity of non-specific cholinesterase. In the liver the increase in total cholinesterase activity also occurred because of an increase in non-specific cholinesterase activity, but was less clear-cut and occurred later (the third day after irradiation).

In animals irradiated 2-3 years before the investigation, an increased concentration of free acetylcholine in brain, liver, and mucosa of the small intestine was noted; but there were no changes in bound acetylcholine. The total cholinesterase activity increased in liver as a result of acetyl cholinesterase increase and non-specific enzymes, and in mucosa of the small intestine only as a result of acetylcholinesterase activity. In brain the total cholinesterase activity decreased as a consequence of a decrease in acetylcholinesterase activity.

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SYNTHESIZING ACTION OF POLYNUCLEOTIDEPHOSPHORYLASE OF MICROCOCCUS LYSODEIKTICUS IN SOLUTION AND IN COACERVATE SYSTEMS

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From the point of view of the study of coacervates as the original systems on the pathway of the origin of life, it was interesting not only to investigate their enzymatic breakdown of nucleic acids (this was done in a previous experiment [1]), but also to accomplish presently the reverse synthesis of polynucleotides from mononucleotides dissolved in the surrounding drops of liquid in equilibrium.

The most suitable enzyme for this purpose seemed to us to be polynucleotidephosphorylase, an enzyme discovered and studied by Ochoa and Grunberg-Manago [2-4], and later by other investigators. By means of this enzyme polymers similar to natural RNA, are synthesized from nucleoside-5-phosphates. It is known that this enzyme is capable of synthesizing a polymer either from a single form of nucleotide, or from the four different nucleotides. The reaction catalyzed by polynucleotidephosphorylase, is expressed by the following equation:

$$nX—R—PP \xrightleftharpoons{Mg^{++}} (X—R—P)_n + nP_{inorg},$$

where R is ribose, PP is pyrophosphate, P_{inorg} is inorganic phosphate and X is the purine or pyrimidine base. The forward reaction is the reaction for synthesizing the polymer, the reverse reaction, the breakdown of the polymer, for which the presence of P_{inorg} is necessary.

METHODS

Substrates. Native preparations of ATP containing ADP were used as substrates, as well as preparations of ADP from the English firm of Light and the Hungarian firm of Reanal. Before the experiments the barium salt of ADP was converted to the sodium salt. The ADP content in all the preparations was determined chromatographically.

For chromatographic separation of the nucleotides a mixture of propanol-ammonia–water (60 : 30 : 10) [5] and Leningrad slow filter paper No. 2 was used. Separation time, 48 hr. After the chromatogram was dried off, the solvent was re-circulated for 72 hr. The chromatograms were examined in UV-light, the spots outlined and cut out. Elution of the nucleotides was carried out with 1 M phosphate buffer, pH 7.0, for 18 hr, at 4°. Five ml buffer was used for the elution of a single spot. Absorption of the eluates was measured on an SF-4 spectrophotometer at 260 mμ. By means of quantitative chromatography the ADP content in the preparations of nucleotide used were determined (Table 1).

Cultivation of the cells. The "small colony" yeast culture, Slonimskii's mutant, strain 59 RA* was cultivated on a wort-agar medium at 30°. After 48 hr the cell growth was washed off with water into a glass bulb. The suspension was centrifuged at 2000 rev/min and the residue washed with 0.5 M NaCl until the turbidity in the supernatant liquid disappeared.

Bakers yeast (obtained from a yeast factory) was washed in the same way with 0.5 M NaCl before isolation of the enzyme.

The bacterial culture M. lysodeikticus N. 2665 Fleming strain** was cultivated at 37° for 24 and 48 hr on meat-peptone agar.

*The culture was kindly given to us by the Laboratory of Prof. M. N. Meisel, Institute of Microbiology, Academy of Sciences of the USSR, Moscow.
**The culture was obtained from the L. A. Tarasevich State Control Institute, Moscow.

TABLE 1. Characteristics of the ATP and ADP Preparations

Preparation	R*	ATP	ADP	AMP
	% of total extinction			
ATP (Native)	—	37,2	35,7	27,1
ATP (Purified) **	12,8	51,3	23,2	12,6
ADP (Light)	15,9	17,2	45,1	21,8
ADP (Reanal)	—	19,0	42,0	39,0

*R is the residue which was located higher than ATP on the chromatogram.

**ATP, purified from Pinorg by Lyubimova's method [6].

Enzyme. Enzyme preparations of polynucleotidephosphorylase were obtained from the "small colony" yeast, bakers yeast and M. lysodeikticus. The enzyme from the bakers yeast and from the small colony yeast was obtained by the method of Brummond et al [7]. Fifty g washed yeast was pulverized with 100 g Al_2O_3 to a fine dust. The powder was taken up in two volumes of 0.15 M KCl and placed in the refrigerator overnight for extraction. All the remaining operations in the first stages of the purification were carried out in accordance with the established technique.

Since, the yield of enzyme was very small (after the first elution with gel the yield was approximately 30 mg), we could not repeat gel purification and limited it to determination of the precipitate used, obtained after 50% saturation of the eluate with $(NH_4)_2SO_4$.

The preparations of polynucleotidephosphorylase which we obtained from the small colony yeast, as well as from the bakers yeast, were definitely active only after long incubation (more than 12 hr). This circumstance would cause great difficulty in the study of the action of these preparations in coacervate systems. Thus, the more active enzyme preparation obtained from M. lysodeikticus was used for inclusion in the coacervate drops.

The enzyme from M. lysodeikticus was obtained according to the method of Beers [8]; the bacteria were removed after 24 hr growth. The cells were washed three times in a solution of 0.14 M NaCl with subsequent centrifugation at 2000 rev/min. After washing and centrifugation 40 g of bacteria (wet weight) were suspended in 130 ml of water; 40 mg of crystalline lysozyme in 474 mg of Tris-buffer in 30 ml of water were added. The lysis was carried out at 37° for 15 min at pH 8.2. All the remaining operations were carried out in accordance with the established technique. The dialyzed and filtered enzyme solution was kept at −18°.

Measurement of the enzyme activity. Beginning with Eq. 1, polynucleotidephosphorylase could be determined by measuring either the forward (synthetic) or reverse (phosphorylation) reactions.

In order to determine the synthetic reaction 52 mg of ATP or ADP were dissolved in 2 ml of water. The solution was adjusted to the necessary pH value with 2 N KOH. The experiments with yeast preparations of the enzyme were performed at pH 8.1; experiments with the bacterial enzyme, at pH 9.5-10.2. Nineteen mg of Tris-buffer, 0.25 ml of 4% $MgCl_2$ and 0.2 ml of 1% versene were added to the reaction mixtures. One ml of enzyme having a protein content of 0.5 to 2 mg was then added. The reaction mixture was incubated at 37°.

After incubation, 0.5 ml of the reaction mixture was withdrawn and the reaction stopped by adding 0.5 ml of 0.25% uranyl nitrate in 2 N $HClO_4$. The precipitate, after standing for an hour at room temperature, was separated by centrifugation at 2000 rev/min for 10 min. The solution was diluted 500 times, the absorption of the solution was measured at 260 mμ.

To determine the activity of the polynucleotidephosphorylase we used the following methods:

The first method consisted of measuring the absorption at 260 mμ of the acid soluble and acid insoluble fractions after precipitation of the reaction mixture with uranyl in $HClO_4$. The change in absorption at 260 mμ of the acid soluble fraction we used as a measure of both the synthetic and the phosphorylytic activity of the enzyme preparations. The change in absorption at 260 mμ of the acid insoluble fraction we used as an indicator for the determination of the synthesizing activity of the bacterial enzyme. The precipitate formed was washed several times with half-strength precipitating agent, then washed with alcohol, and dissolved in 3 ml 0.1 M Tris-buffer, pH 8.0.

TABLE 2. Comparative Study of Deviations in the Use of the First Method for the Determination of Enzyme Activity

$\frac{E_{280}}{E_{260}}$	Amt. of protein, mg/ml	Substrate and pH of the reaction mixture	Time of incubation, hr	$E_{average}$	$\Delta E_{average}$	Substrate used, % of initial	Standard deviation	No. of expts.
0.63	2,8	ADP*	0	0,186	—	—	±0,019	20
			1	0,164	0,022	29,0	±0,020	15
		42% ADP,	2	0,160	0,026	34,2	±0,015	13
		pH 9.5-10.3	3	0,150	0,036	47,3	±0,015	12
			4	0,147	0,039	54,3	±0,015	12
0,84	0,56	ATP**	0	0,419	—	—	±0,014	12
			1	0,399	0,020	13,3	±0,015	11
			2	0,381	0,038	25,3	±0,021	12
		36% ADP,	3	0,396	0,023	15,3	±0,012	11
		pH 8,1	4	0,394	0,025	16,6	±0,017	9
0,73	0,81	ATP**	0	0,363	—	—	±0,017	12
			1	0,344	0,019	12,6	±0,016	12
			2	0,343	0,020	13,3	±0,016	10
		36% ADP,	3	0,331	0,032	21,3	±0,016	12
		pH 8,1	4	0,347	0,016	16,6	±0,019	10

*ADP from Reanal.

**ATP, native product.

The solution was diluted 10 fold and its optical density measured. Simultaneous absorption measurements at 260 mμ for the acid soluble and acid insoluble fractions enabled us to obtain a balance of the nucleic acid phosphorus. As a unit of enzyme we took that quantity which catalyzed a change in nucleic acid phosphorus content of 1 μmole during 1 hr at 37°.

The second method was based on the determination of P_{inorg} and P_{tot} and subsequent calculation of a balance based on equation 1.

Orthophosphate was determined by the Fiske-Subarrow method. Nucleic acid phosphorus was determined by absorption at 260 mμ. For conversion of the data the hyperchromic effect was taken into account [8]; the molar extinction for AMP, ADP and ATP was assumed to be 15.4 [9].

For determination of the nucleic acid phosphorus of the acid insoluble fraction the precipitate was washed, as described above, dissolved in 5 ml 0.5 N $HClO_4$ and hydrolyzed for 15 min at 100°. The protein which precipitated was removed by centrifugation. The supernatant liquid was diluted 10 fold and its optical density at 260 mμ determined.

Determination of protein. Protein was determined by Lowry's method [10]. The data obtained were converted according to a calibration curve, obtained for human serum albumin.

RESULTS AND DISCUSSION

Beer's data on the presence of a polynucleotidephosphorylase in a lysate of M. lysodeikticus was obtained in bacterial cells, cultivated on agar media with glucose. Thus, prior to attempting to obtain enzyme preparations from cells of M. lysodeikticus cultivated on meat-peptone media, we carried out preliminary experiments to determine the activity of polynucleotidephosphorylase in lysates of these bacteria. Our experiments verified the presence of this enzyme in the investigated bacteria.

Using the procedure of Beers [11] we isolated 5 active enzyme preparations and examined the scattering of data when the first method for determination of enzyme activity was used (Table 2). It was found that the specific activity varied from 1.43 to 6.50.

The data obtained indicated that the first method for the measurement of synthetic activity was completely acceptable, since the average scattering of the data did not exceed 5%.

TABLE 3. Partial Purification of Polynucleotidephosphorylase from M. lysodeikticus (from 40 g of washed cells)

Stage of purification	Vol. ml	Activity, units	Protein, mg	$\frac{E_{280}}{E_{260}}$	Specific activity, units/mg protein	Yield, %
Extract	200	—	880	0,58	—	100
Precipitate after 50% saturation with $(NH_4)_2SO_4$	40	227,4	544	0,74	0,42	62
Precipitate after 50% saturation with acetone	27	204,1	75,6	0,63	2,70	8,5

TABLE 4. Phosphorus Balance Calculated from the Determination by the First and Second Methods of Polynucleotidephosphorylase Activity on ADP Preparations

No. of expt.	Time of incubation, hr	Nucleic acid						Total* P, μmoles
		acid soluble fraction		acid insoluble fraction		acid soluble fraction		
		E	P, μmoles	E	P, μmoles	E	P, μmoles	
1	0	0,296	9,60	0,000	0,000	—	—	9,600
	1	0,264	8,60	0,125	0,488			9,088
	2	0,224	7,30	0,341	1,330			8,630
	3	0,202	6,60	0,540	2,100			8,700
	4	0,179	5,60	0,712	2,780			8,380
2	0	0,156	5,07	0,000	0,000	—	—	5,070
	1	0,146	4,75	0,036	0,104			4,854
	2	0,146	4,75	0,109	0,318			5,068
	3	0,132	4,29	0,196	0,570			4,860
	4	0,112	3,97	0,262	0,746			4,716
3	0	0,180	5,85	0,016	—	—	—	5,850
	1	0,165	5,36	0,148	0,370	—	—	5,730
	2	0,155	5,04	0,291	0,805			5,845
	3	0,142	4,62	0,398	1,110			5,730
	4	0,134	4,35	0,482	1,380			5,730
4	0	0,225	7,30	0,034	0,100	—	—	7,400
	1	0,190	6,10	0,043	0,125	—	—	6,225
	2	0,182	5,90	0,063	0,183			6,083
	3	0,183	5,90	0,133	0,386			6,286
	4	0,173	5,60	0,290	0,843			6,243
5	0	0,207	6,72	0,083	0,200	—	—	6,920
	2	0,169	5,48	0,320	0,930			6,410
	3	0,164	5,32	0,375	1,090			6,410
6	0	0,175	5,690			0,197	3,42	5,690
	1	0,160	5,20			0,225	3,94	5,720
	2	0,145	4,71			0,220	3,94	5,230
	3	0,140	4,55			0,267	4,71	5,320
	4	0,135	4,38			0,265	4,71	5,150
7	0	0,145	4,72			0,154	2,09	4,720
	1	0,138	4,48			0,182	2,53	4,920
	2	0,132	4,28			0,183	2,53	4,720
	3	0,132	4,28			0,182	2,53	4,720

*Micromoles of nucleic acid phosphorus from the acid soluble fraction + μmoles nucleic acid phosphorus from the acid insoluble fraction (or in experiment 6, increase in μmoles P_{inorg}, equivalent to the increase in nucleic acid phosphorus of the acid insoluble fraction).

TABLE 5. Synthesis of Polyadenylic Acid on Coacervate Suspension

Time of incubation, hr	Acid soluble fraction			Acid insoluble fraction			P_{inorg}		
	E	P	ΔP	E	P	ΔP	E	P	ΔP
		micromoles			micromoles			micromoles	
0	0,367	11,93	—	0,050	0,162	—	0,242	4,260	—
3	0,345	11,22	0,710	0,298	0,968	0,806	0,279	4,980	0,720

Characteristics of the enzyme preparation obtained by Beers' method from M. lysodeikticus are presented in Table 3.

It is evident from this table that the yield was 8.5%; this coincides quite well with data in the literature dealing with the preparation of the enzyme from other material.

Results presented in Table 4 indicate that the use of both the first and second methods for the determination of enzyme activity is possible, since a stoichiometric relationship existed between the nucleic acid phosphorus of the acid soluble and acid insoluble fractions on the one hand, and the nucleic acid phosphorus and P_{inorg} of the acid soluble fractions on the other hand.

In choosing ingredients for coacervate systems, we were directed to the fact that ribonucleic acid is associated with alkaline protein in cellular structures. According to the data of Liebl [12], a coacervate may be obtained from histone of the thyroid gland and RNA at a pH of 10. Turning our attention to these data, we attempted to accomplish the synthesis of polyadenylic acid in a coacervate consisting of RNA and histone.* Polynucleotidephosphorylase was included in this coacervate.

We first studied the influence of histone and RNA on the activity of polynucleotidephosphorylase. For this purpose, either 26 mg RNA, or histone in such quantity that its concentration in the medium was 1%, was added to the reaction mixture described above for the determination of enzyme activity. The determination of ΔE in experimental (with the indicated additions) and control samples (without any addition) are shown below:

Added substance	Control	Experimental
RNA	0.028	0.030
Histone	0.060	0.040

These data indicate that RNA and histone have no significant effect on the activity of polynucleotidephosphorylase. This fact established, we proceeded to include the enzyme in the coacervate drops. In order to obtain coacervates, 26 mg of RNA was added to 2 ml substrate mixture, and 30 mg histone was added to 1 ml enzyme. By combining these solutions a coacervate suspension was formed, in which the reaction was carried out. After 3 hr of incubation the nucleic acid phosphorus of the acid soluble and acid insoluble fractions and P_{inorg} were determined (Table 5).

The data of Table 5 demonstrate that in RNA and histone coacervates the synthesis of polyadenylic acid takes place.

SUMMARY

A study of the action of polynucleotidephosphorylase preparations obtained from cells of M. lysodeikticus by Beers' method, showed that macromethods of mineral and nucleic acid phosphorus determination could be used for the measurement of enzyme activity.

The presence in the reaction medium of RNA or histone did not significantly effect the enzyme activity. Synthesis of polyadenylic acid occurred in coacervates from RNA and histone.

*The thyroid gland histone obtained at the Biological Institute in Prague, was kindly furnished by Prof. W. Liebl.

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GLUTAMINE BIOSYNTHESIS IN THE TISSUES OF AN ANIMAL ORGANISM

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In recent years a large number of works devoted to the study of biosynthesis of glutamine and asparagine has been published. One of the possible ways of biosynthesizing amides is the transamidation reaction postulated by Mardashev and Lestrovaya [1, 2], which in recent years has undergone experimental verification in a number of laboratories.

In 1953, Sheffner and Grabow [3] observed the formation of glutamine in yeast grown on a medium containing glutamic acid and asparagine, but in the absence of Mg^{++}, i.e., under conditions where no formation of glutamine takes place if the free ammonium is the donor of the glutamine amide group. In the same year, Denes [4] reported isolation from Lupinus albus shoots of an enzyme which effects asparagine synthesis from aspartic acid via utilization of the glutamine amide group, but without free ammonium. Later, Levintow and co-workers [5-7] demonstrated that HeLa cells which are not capable of forming glutamine, form asparagine upon the addition to the medium of a glutamine labeled N^{15} in its amide group and C^{14}-aspartic acid; the N^{15} content in its amide group corresponds to the N^{15} content in the amide group of added glutamine. Since, in the presence of $N^{15}H_4Cl$ in the medium, N^{15} is not included in asparagine, a conclusion has been arrived at that the glutamine amide group can be directly employed for asparagine synthesis without the intermediate stage of free ammonium formation. It should be, however, pointed out that the latter data relate not to the amide group of free asparagine, but to an amino acid which enters into the composition of cellular protein. Bauerova and Schorm [8] demonstrated that, upon infiltration into the Brassica napus shoots of glutamine and asparagine labeled in the glutamine amide group, the amide nitrogen of asparagine emerges in large quantities in the glutamine amide group, and the amide N of glutamine becomes incorporated in the amide group of asparagine. The authors assume that at the basis of glutamine formation from asparagine lies transamidation, because isopropylphenylcarbamate (IPPC), which inhibits formation of glutamine from glutamic acid and ammonium, does not affect the increase of glutamine content following infiltration of asparagine in the plant.

However, Webster and Varner [9], using C^{14}-aspartic acid in experiments with extracts from pea-shoots and wheat-germs, were unable to elicit asparagine formation when the glutamine amide group served as a nitrogen source. The data of Hsu Ting-seng [10] indicates that in the liver slices of rats and pigeons glutamine forms at an equal rate in the system glutamic acid + asparagine and in the system glutamic acid + NH_4. On the basis of obtained data, the author of the latter work thinks that there is no reamidation reaction present. Although the data of Hsu Ting-seng are beyond doubt and have been confirmed in our laboratory, they do not serve as indisputable proof of the absence of transamidation reaction, since the proof of a glutamine-synthetase reaction does not refute alternate possibilities.

For the elucidation of these possibilities we decided to investigate at each stage of purification all three processes using the Spek method of purification of glutamine-synthetase: the glutamine synthesis in the system glutamic acid + NH_3, glutamic acid + asparagin, and splitting of asparagine (i.e., the presence of asparatase).

METHODS

The experiments were carried out with slices and homogenates of pigeon's liver with slices of rat's liver. Two hundred mg of the tissues were added to the test material two ml in volume, containing phosphate, acetate or Tris-buffer and substrates in concentration of 100 micromoles per test. The NH_4Cl concentration in a number of experiments comprised 20 micromoles per test. Incubation lasted 90 min at 37° in an atmosphere of O_2 or air. When necessary, the tests were then acidified up to pH 5.0, boiled for 3 min, and the proteins removed via centrifigation.

We gave up the previously used method [11] of glutamine determination, because it included ammonium determination as per Conway which was not sufficiently accurate, especially at low magnitudes of amide synthesis and large quantities of added ammonium.

The tests were chromatographed in the system butanol–85%-formic acid–water (75 : 15 : 10) with 5-fold passing of the solvent through Watman paper No. 2 or No. 4. The quantitative determination of amide and dicarbonic acids was carried out by means of photometric examination of ninhydrin-copper complexes in a SF-4 spectrophotometer at 530 micromicrons [12]. Ammonium was distilled off under isothermic conditions as per Conway (0.1 per H_2SO_4), and determined following nesslerization on a CF-4 spectrophotometer at 420 mmc. The Nessler reagent was diluted (1 : 4) in order to reduce the magnitude of the reagents control tests.

A purified enzymic system which would affect glutamine synthesis upon the use of various substrates was carried out according to the Speck method [13], developed for the purification of glutamine-synthetase. The purification process included the following stages: 1) extraction of an acetone powder of pigeon's liver with 0.2 M $NaHCO_3$ with subsequent dialysis against 0.15 M NaCl – 0.005 M $NaHCO_3$ (fraction 1, dialyzed preparation); 2) precipitation of proteins after dialysis with 0.2 M Na acetate, pH 4.2. The sediment was dissolved in 0.2 M $NaHCO_3$ and 0.15 M NaCl – 0.005 M $NaHCO_3$, and the solution dialyzed against 0.15 M NaCl – 0.005 M $NaHCO_3$ (fraction 2, isoelectric precipitation); 3) precipitation of the proteins of fraction 2 with ethanol (final concentration 22 vol.%). The precipitate was dissolved in 0.15 M NaCl – 0.005 M $NaHCO_3$ (fraction 3, precipitation with 22% ethanol); 4) precipitation of proteins of fraction 3 with a further increase of ethanol concentration (final concentration 43 vol.%). The precipitate was dissolved in 0.15 M NaCl – 0.005 M $NaHCO_3$ (fraction 4, and subsequent precipitation with 43% ethanol).

All above-described fractions as well as the solutions and sediments, which are rejected by Speck in the purification process, were checked in regard to the glutamine synthesis in a system containing 0.05 M acetate buffer, pH 5.5 and in micromoles: $MnCl_2$, 15; ATF, 3; glutamate Na, 50; asparagine or NH_4Cl, 50; 0.4 ml of the enzymic preparation. The volume of the test-specimens was one ml, incubation 90 min at 37°. The formation of glutamine from glutamic acid and NH_3 as well as from glutamic acid and asparagine was observed in all above-mentioned fractions

Upon determination of the enzymic activity of separate fractions, according to the synthesis of hydroxamic acid from glutamic acid and hydroxylamine, we obtained values analogous to the ones cited by Speck [13]. The enzymic activity of the purified fractions was preserved without any substantial change for a period of 2-3 weeks under storage conditions at 2°. A more extended storage led to the reduction of enzymic activity. Heating of the preparations to 50-55° for 10 min completely inhibited the enzyme.

RESULTS AND DISCUSSION

The data in Table 1 illustrate the glutamine synthesis in the homogenates of the pigeon's liver. Since the glutamine synthesis in the specimens which contain asparagine and glutamic acid could take place not as a result of the transfer of amide group directly on glutamic acid with formation of glutamine, but at the expense of NH_3 formed during the hydrolysis of the added asparagine [10]; we added ammonium to the glutamic acid in a number of experiments in the amount corresponding to the quantity of ammonium formed at the hydrolysis of added asparagine, under given experimental conditions. As shown by our determinations which coincide with the data of Hsu Ting-seng [10], the NH_3 concentration for a pigeon's liver under given conditions equalled ~15-20 micromoles on the average.

TABLE 1. Glutamine Synthesis by Homogenates of Pigeon's Liver, Volume of Test 2 ml; Asparagine and Glutamic Acid āā 100; NH_4Cl – 20 and 100 Micromoles per Test; Homogenate of Pigeon's Liver 200 mg of Tissue per Test; 0.05 M Phosphate Buffer, pH 7.4

Composition of the test	No. of the expt.									
	1	2	3	4	5	6	7	8	9	10
Asparagine + glutamic acid	11,7	9,6	1,9	4,7	2,1	8,4	12,6	4,9	9,2	10,1
Glutamic acid + NH_4Cl, 20 micromoles	13,2	9,8	2,1	7,1	3,3	3,8	12,4	3,5	8,9	9,2
Glutamic acid + NH_4Cl, 100 micromoles	12,9	10,3	4,4	—	—	—	13,0	2,9	13,0	15,1

TABLE 2. Glutamine Synthesis with Acetone Preparations of Pigeon's Liver. Volume of Tests, 2 ml; Asparagine and Glutamic Acid āā 100; NH_4Cl 20; ATF 1.8; $MgSO_4$ three micromoles per Test; Acetone Preparation 60 mg; Tris Buffer, pH 7.6 (acetone preparations obtained from various pigeons)

Composition of tests	No. of the expt.				
	1	2	3	4	5
Asparagine + glutamic acid	1,3	1,1	1,0	2,34	1,04
Glutamic acid minus NH_4Cl	0,84	1,1	0,7	1,04	0

TABLE 3. Glutamine Synthesis in the Slices of Rat's Liver. Volume of Tests 2 ml; Asparagine and Glutamic Acid āā 100; NH_4Cl 20 micromoles per Specimen; Liver Slices 200 mg; 0.05 M of phosphate Buffer, pH 8.0

Composition of tests	No. of the expt.			
	1	2	3	4
Asparagine + glutamic acid	1,4	1,19	1,0	0,8
Glutamic acid + NH_4Cl	1,3	1,20	0,7	0,9

It can be seen from cited data that glutamine synthesis in pigeon's liver proceeds with sufficient intensity and does not depend on the fact whether asparagine or NH_4Cl is present as a donor of the amide group. However, our attention is attracted to the non-uniform character of the obtained data: whereas in five experiments (2, 3, 7, 9) the amide formation proceeded uniformly in both cases, in three experiments (1, 4, 5) it was NH_3 which was preferably used as a donor of the amide group, and in three experiments (6, 8, 10) asparagine was the donor of choice.

Analogous results have been obtained in our work with acetone preparations of pigeon's liver (Table 2) and also with slices of rat's liver (Table 3).

In the analysis of obtained data it is necessary to take into consideration the fact that, as shown by control experiments, the hydrolysis of glutamine in the presence of 20 micromoles of NH_4Cl per specimen is considerably inhibited (by 20-30%); on the other hand, in the tests where asparagine is present as the donor of the amide group, the hydrolysis of asparagine proceeds gradually with the liberation of 1.5 to 2 micromoles of NH_3 every 10 min; we should, therefore expect that the effect of glutaminase I in these tests would be more marked. If it is so, then the real magnitudes of glutamine synthesis in the system asparagine + glutamic acid should be apparently higher. The fact that in the presence of 100 micromoles of NH_4Cl large quantities of glutamine are frequently formed can possibly be explained by the even more pronounced inhibition of glutamine hydrolysis in these tests. However, the attempt to imitate a more precise control in the system asparagine + glutamic acid via addition of NH_4Cl to glutamic acid in fractional doses (two micromoles every 10 min) brought no definite results.

The cited data does not enable us to decide whether a direct transamidation of asparagine on the glutamic acid takes place followed by formation of glutamine, or to deduce that asparagine represents only a donor of NH_3 (which is formed as the result of the action of asparatase) which in its turn is utilized for the amidation of glutamic acid. The most serious objection to the transamidation reaction are the data that the inhibitors of glutamine-synthetase (NaF and crystal-violet) as well as aerobic conditions equally inhibit the formation of glutamine at the expense of asparagine. However, the possibility is not excluded that these substances may also inhibit the enzyme which effects transamidation, and that the sources of energy of the ATP type are, as we know, essential also in some reactions of transamidation (the utilization of glutamine for the formation of glucosamine or amination of xanthonic acid). In this connection, of special interest is the mechanism of synthesis of the amido group of codehydrogenase I and II [14]. The amido group of nicotinamide is formed at the nucleotide stage from NH_3 or directly from the amido group of glutamine, both reactions are in need of ATP.

TABLE 4. Glutamine Synthesis by Means of Purified Enzymic Preparations Obtained from Pigeon Liver. Volume of tests 1 ml; asparagine and glutamic acid $\overline{aa}$ 50; $MnCl_2$ 15; ATF 3 micromoles per test; enzymic preparation 0.4 ml (0.39 mgN); 0.05 M acetate buffer, pH 5.5

No. of test	Composition of test	Amt. of NH_3 in 1 ml of test of test, μM	Synthesized glutamine, μM
1	Enzymic preparation	0.50	0
2	Same + asparagine	0.92	0
3	Same + glutamic acid	0.46	0.21
4	" + glutamic acid + asparagine	0.78	0.33
5	" + glutamic acid + NH_4Cl, 0.5 μM	0.83	0.84
6	" + " " + NH_4Cl, 0.33 μM	0.65	0.57
7	" + " " + NH_4Cl 0.13 μM	0.70	0.31
8	" + " " + NH_4Cl 0.5 μM + asparagine	–	0.84
9	Preparation + glutamine	4.10	–
10	Asparagine	0.50	–
11	Glutamine	3.57	–
12	Glutamic acid	0.37	–
13	Glutamic acid + asparagine	0.55	–
14	Glutamic acid + NH_4Cl, 0.33 μM	0.66	–
15	Glutamic acid + NH_4Cl, 0.5 μM	0.85	–

It seems to us that the solution of the problems whether a transamidation reaction exists parallel with the amidation reaction can be effected only upon the use of specific inhibitors of enzymic systems and purified enzymic preparations. In this connection, considerable interest was created by the report of Bauerova and Shorm [8] that, in the shoots of rape, IPPC inhibits the synthesis of glutamine from glutamic acid and NH_3, whereas no effect was exerted by IPPC on the increase of glutamine content after the infiltration of asparagine. However, under conditions of our experiments with the homogenates and purified preparations of pigeon liver, IPPC exerted no such effect either in the concentration employed by Czech authors (2 μM/gm tissue), or in a 3.5-fold concentration. Lerman [15] obtained data on a virtually complete and unhampered aspartase inhibition from the serum of a guinea pig by DL-alpha-methylaspartate. Since in one of the purified preparations, which we had obtained from pigeon liver the aspartase enzyme was present, the synthesis of glutamine in the system asparagine + glutamic acid could have been imitated by two enzymes: aspartase and glutamine-synthetase. It turned out that in the presence of DL-alpha-methylaspartate (in concentration equimolar to the added substrates) the biosynthesis of glutamine from asparagine and glutamic acid was considerably reduced or practically absent. It was also found that DL-alpha-methylaspartate under our experimental conditions inhibits the glutamine-synthetase to a certain extent.

We obtained a purified enzymic preparation from pigeon's liver which, as we found out, contained no aspartase I (Table 4, tests 1, 2, 10) or glutaminase (Table 4, tests 1, 9, 11). However, despite the absence of aspartase activity, a certain though slight synthesis of glutamine was observed in the system asparagine + glutamic acid (Table 4, test 4).

We measured the NH_3 content in all our reagents in order to elicit the possible sources of NH_3 in this system. As seen in Table 4, the preparation itself (test No. 1) contains 0.5 μM of NH_3 and glutamic acid – 0.37 μM of NH_3. Since both components are present in the system glutamic acid + asparagine, as well as in the system glutamic acid + NH_3, the supplementary source of ammonium in test No. 4 is only asparagine, 50 μM of which contain 0.5 μM of NH_3. However, upon addition of 0.5 μM of NH_3 to glutamic acid (test No. 5), 0.84 μM of glutamine are formed, i.e., more than twice in test 4. On the other hand, to produce 0.3 μM of glutamine, only 0.13 μM of NH_3 were needed for the test containing glutamic acid + NH_3 (test No. 7). Hence, we assumed that the large quantities of asparagine in test 4 may have inhibited glutamine synthesis, possibly as an analog which competes with glutamine for the enzyme. Upon staging a corresponding experiment (test No. 8), it turned out that in this case less glutamine

is formed than could be expected from the total amount of tests 4 and 7. In favor of this assumption are also the data obtained in experiments with homogenates of pigeon and rat liver.

In working with purified preparations from pigeon liver, we were unable to elicit formation of asparagine from aspartic acid and glutamine, i.e., a reverse reaction which would attest to the existence of the transamidation process.

SUMMARY

The mechanism of glutamine formation in pigeon and rat liver was investigated in the systems glutamic acid + asparagine and glutamic acid + NH_3. It was shown that the rate of glutamine formation in both systems is similar and varies only within the range of experimental error.

The investigation of glutamine synthesis was carried out also with various fractions obtained in the process of purification of glutamine-synthetase as per Speck. The obtained enzymic preparations sometimes contained no aspartase, and the glutamine synthesis which takes place in the presence of these substances and glutamic acid is determined by the presence of free NH_3 in the utilized reagents. The glutamine synthesis does not take place in a preparation possessing aspartase activity when DL-alpha-methylaspartase is added, since the latter inhibits aspartase and partially also the glutamine synthetase.

Thus the transamidation reaction in the pigeon's liver is imitated via two enzymes—aspartase and glutamine synthetase.

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THE "TEMPERATURE EFFECT" AND MACROMOLECULAR STRUCTURE OF HIGH-POLYMER RIBONUCLEIC ACIDS OF VARIOUS ORIGIN

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The history of investigation of the macromolecular structure of native ribonucleic acids (RNA) comprises only a few years. The first results in this direction have been achieved on the high-polymer RNA from the tobacco mosaic virus (TMV). Isolated via definite methods, the RNA from TMV turned out to be possessing its own activity—infectiousness [1, 2]; it, therefore, attracted attention immediately as a definite native RNA.

Through the works of the Sinsheimer laboratory [3] which had somewhat anticipated the discovery of the infectious character of the high-polymer RNA of TMV, and subsequent detailed investigations of Gierer [4] the problem of the molecular weight (m.w.) of the native RNA TMV molecules and partially also the form of this RNA molecule in solution was thoroughly studied. The investigations in our laboratory [5-7] and in the Doty laboratory made possible the elicitation of a number of characteristics of the internal structure of molecules of RNA virus in solution. The most important result of these works was the establishment of a definite structural regularity in the macromolecules of the RNA virus [6, 7, 9].

It has been elicited in our laboratory that upon heating of solutions of the infectious TMV RNA, the RNA molecules undergo a structural transition of the type "order-disorder" which is manifested in a staggered increase of the reduced viscosity with a simultaneous decrease of the sedimentation constant [6]. This phenomenon—the increased viscosity within a definite temperature interval—was named the "temperature effect." The temperature effect of infectious RNA from TMV was used in our subsequent work jointly with the Bresler* laboratory [7] and Dvorkin** [10] as a clue to the investigation of its molecular structure in solution. On the basis of the study mainly of the infectious viral RNA, it became possible to obtain a definite idea of the primary, secondary, and tertiary structures of high-polymer RNAs in a solution [11].

At the present time it is known that in addition to viruses the high-molecular RNAs are contained in the cells of all living organisms. These cellular high-molecular RNAs represent the compound part of ribosomes (ribonucleoproteid particles) of cells. In recent years, such RNA preparations have been isolated from cells and tissues of various origin—animal [12-15], plantar [12], and bacterial [16, 17].

Their study in solution showed that in regard to a number of physicochemical properties—the form and compact character of the particles, the ratio of molecular weight, sedimentation constant, characteristic viscosity, magnitude of hypochromism and the number of intramolecular hydrogen bonds—the cellular RNAs in solution are analogous to the infectious viral RNA [8, 9, 12-16]. Moreover, Cox and Littauer [18] reported on the structural transition of RNA macromolecules from E. coli under the effect of reduced ion strength, thus attesting to the presence of well-regulated structures in the macromolecules of high-polymer RNA of cellular origin. Somewhat later, jointly with Mil'man*** [19] we elicited a structural transition in a high-molecular RNA of animal origin (from the liver and cells of ascitic hepatoma of rats) as the result of heating; the studied RNA showed under these circumstances a temperature effect similar to the one in viral RNA. All this, on the whole, permitted us to assume that the macromolecular structure of cellular RNAs in solution is analogous in its basic characteristics to the structure of the infectious viral RNA under similar conditions, and that the principles of macromolecular organization which had been formulated for viral RNA in solution [11] are generally applicable also to high-polymer RNAs of cellular origin. At

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the same time, certain difference was observed in the molecular weights between various cellular RNAs on the one hand and viral RNA, on the other. Whereas infectious viral RNA is characterized by monodispersion particles with mol. wt. $\sim 2 \cdot 10^6$, the cellular RNA preparations showed as a rule two high-molecular components of somewhat lower mol. wt. [12-17].

In the present work we attempted to approach experimentally the problem of the similarity and differences in the macromolecular structure of high-molecular RNAs in solution by means of direct comparative study, under identical conditions and methods, of RNA preparations of various origin.

METHODS

Material and Methods

As the source material for obtaining preparations of high-molecular RNAs, we employed: the TMV preparation obtained from young mosaic tobacco leaves by means of fractionation of the juice with ammonium sulfate and subsequent three-fold preparatory ultracentrifugation; a bacterial mass of E. coli (B strain) grown on a synthetic medium with a yeast extract at 37° (with aeration) for 14 hr (end of logarithm phase); green week-old pea shoots; rabbit's bone marrow extracted immediately after killing the animal.

The RNA content of the preparations and RNA concentration in the solutions were determined spectrophotometrically [20]. Tests with the sedimentation of high-polymer RNAs were carried out in a Spinko analytical rotor ultracentrifuge, model E.*

The specific viscosity (η_{sp}) was measured in Ostwald's viscosimeter processed via Wobser water-ultrathermostat. The curves of the sp.vis. temperature dependence were taken by means of measuring the viscosity of the same RNA solution at various temperatures (from 20 to 80-90°) with 10° intervals. In case of the presence of RNA polysaccharid admixtures, also when the necessity arose of ascertaining whether the viscosity had been caused by RNA only, the RNA solution was processed (after taking the curve of temperature dependence) with crystalline ribonuclease (English preparation made by Light & Co) and the viscosity was measured again under the same conditions. The solutions of RNA preparations, freed of admixtures, revealed,following the effect of ribonuclease,practically negligible traces of sp. vis. In the case of contaminated RNA preparations, the sp. vis. of the solutions processed with ribonuclease could be fully related to the presence of non-nucleic admixtures. Therefore, in order to obtain the true sp. vis. of RNA, in these cases we subtracted from the originally measured sp. vis. in the solution, the sp.vis. obtained after the action of ribonuclease on the solution, and this was accepted as the sp. vis. of the non-nucleic "background" (the temperature dependence of the sp. vis. background had a character of a more or less uniformly descending curve).

Extraction of High-Polymer RNAs

Preparations of high-polymer RNAs were obtained by means of phenol deproteinization [1, 21]; the initial material was processed (virus, bacterial protoplasts, animal, or plantar tissues) with a mixture of equal volume of water-saturated (90%) phenol and 0.01 M buffer (benzoate or Tris), pH 7.4 to 9.0 in the presence of versen at room temperature The RNA which had passed into the aqueous layer, after separation of the phases in the cold was subjected to two more deproteinizations with phenol. The high-molecular RNA was separated from the low-polymer RNA fractions and from DNA admixtures via its precipitation in the cold with 1.5 M NaCl [22, 23].

RNA extraction from TMV. The high-polymer infectious RNA was obtained from TMV basically by the Gierer-Schramm method [1] with previously described modifications [24], with the exception that as a buffer for the virus solution we used the 0.01 M benzoate buffer, pH 8, containing 0.001 M versen, and the stirrring up with phenol was done at room temperature. Following final centrifugation on the suspension at 0° the aqueous phase was collected, RNA was precipitated with three volumes of alcohol in the presence of Na acetate, the precipitate was dissolved in water, and an equal volume of 3 M NaCl was added. The precipitate which had fallen out after keeping the solution in the cold during the night, was reprecipitated with alcohol, dissolved in a phosphate buffer, pH 7.3, micron 0.1 containing versen in a 0.01 M concentration, and the solution stored in a frozen state at minus 70°.

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RNA extraction from E. coli. In order to obtain RNA from E. coli we used basically the procedure described by Littauer and Eisenberg [16]. Bacterial cells, collected at the end of the logarithmic phase of growth, were preliminarily transferred into protoplasts via lysozyme in a Tris-buffer; they were then lyzed by adding versen, and the lyzate immediately processed with phenol at room temperature. The RNA precipitation with alcohol, reprecipitation of the high-polymer RNA with NaCl, dissolving it in a working buffer (phosphate, micron 0.1 with 0.01 M of versen), and the storage of solution were accomplished in an identical manner as in the case of viral RNA.

Extraction of RNA from pea-shoots. About 15 gm of fresh shoots were homogenized for two minutes in the cold in a speed grinder (with a razor) in 100 ml of 0.01 M Tris-buffer, pH 7.3 containing 0.01 M $MgCl_2$. We then added to the homogenate 150 ml of freshly distilled water-saturated phenol and 50 ml of 0.01 M versen, pH 8. The mixture was stirred for 30 min at room temperature, then 50 ml of chloroform were added and the stirring continued for another 30 min. After centrifugation at 0°, the lower phenol-chloroform layer with the transferred pigment, as well as the intermediary layer, were discarded, while the colorless transparent upper layer (aqueous phase containing RNA) was collected and subjected to two more reproteinizations (1 hr and 30 min) with phenol and the addition of chloroform in the middle of its deproteinization. The RNA was precipitated with alcohol from the aqueous phase after the last centrifugation, redissolved in water, the high-polymer RNA was then reprecipitated with NaCl, the residue reprecipitated with alcohol again, dissolved in a buffer, and the solution stored at minus 70°. In order to separate the polysaccharides, the frozen solution was thawed out in the cold and centrifuged at 0° for an extended period of time until the polysaccharide had precipitated. The centrifugate was separated, frozen again, then thawed out and centrifuged again. This procedure ensured the proper purification of RNA solutions from the concomitant polysaccharides and had no effect on the RNA quality.

Extraction of RNA from a rabbit's bone marrow. Freshly-separated bone marrow (~ 15 gm) was homogenized for five minutes in the cold in a speed microgrinder with razor) in the following mixture: 45 ml of 0.01 M versen, pH 8; one ml 1 M benzoate buffer, pH 8; 35 ml of freshly distilled water-saturated phenol and 10 ml chloroform. Following homogenization, we added 100 ml of 0.01 M versen, pH 8 and 100 ml of phenol; the mixture was stirred for one hour at room temperature. All subsequent procedures were carried out exactly as in the case of RNA extraction from pea shoots. Polysaccharide admixtures were separated via freezing and prolonged centrifugation at 0°.

EXPERIMENTAL PART AND DISCUSSION OF RESULTS

The Molecular Weights of High-Polymer RNAs

As mentioned previously, in contrast to RNA from TMV, the cellular ribosomal RNAs are bipartite [12, 17]. The two components of the cellular high-polymer RNA are clearly seen on the sedimentation diagrams in the form of two monodispersion peaks, with different sedimentation rates and, consequently, different molecular weight. The bicomponent nature of the high-polymer RNA cells is apparently, to a considerable extent, the reflection of the fact that the ribosomal particles consist of two unequal sub-units and that, correspondingly, the larger sub-unit contains the RNA molecule with the larger molecular weight, while the smaller sub-unit contains an RNA molecule of lower mol. wt. [17].

The comparative study of high-polymer RNAs of various origin undertaken in this work was aimed in particular to a certain clarification of the problem relating to the comparative size of various molecules of cellular RNAs and their mol. wt. especially in comparison to the mol. wt. of the viral RNA.

Fig. 1 represents sedimentation diagrams of the studied preparations of the high-polymer cellular RNA: from E. coli (A), rabbit's bone marrow (B) and pea shoots (C). All tests were carried out at 20° in a phosphate buffer, pH 7.3, micron 0.1 at 50740 vol/min. Simultaneously, under identical conditions, tests were carried out with RNA from TMV (sedimentation diagram of RNA from TMV under these conditions were published earlier [6, 7, 24]).

In Table 1 are shown the values of sedimentation constants ($S_{20,w}$) of studied RNAs in a phosphate buffer, micron 0.1 at 20°, extrapolated in relation to infinite dilution.

In order to calculate molecular weights from $S_{20,w}$ obtained under given conditions, we employed the indirect approach based on the fact that the mol. w. of RNA from TMV and both RNA components from E. coli had been previously firmly established in the literature [4, 8, 17]. The mol. w. of RNA from TMV, according to the most direct measurements and calculated directly from the percentage content of RNA in the virus, equals $2.1 \cdot 10^6$ [4, 8]. The mol. w. of the ribosomal RNA from E. coli, obtained recently via careful measurements and calculated directly from the percentage content of RNA in each of the two ribosomal sub-units of E. coli equals, respectively, for the two

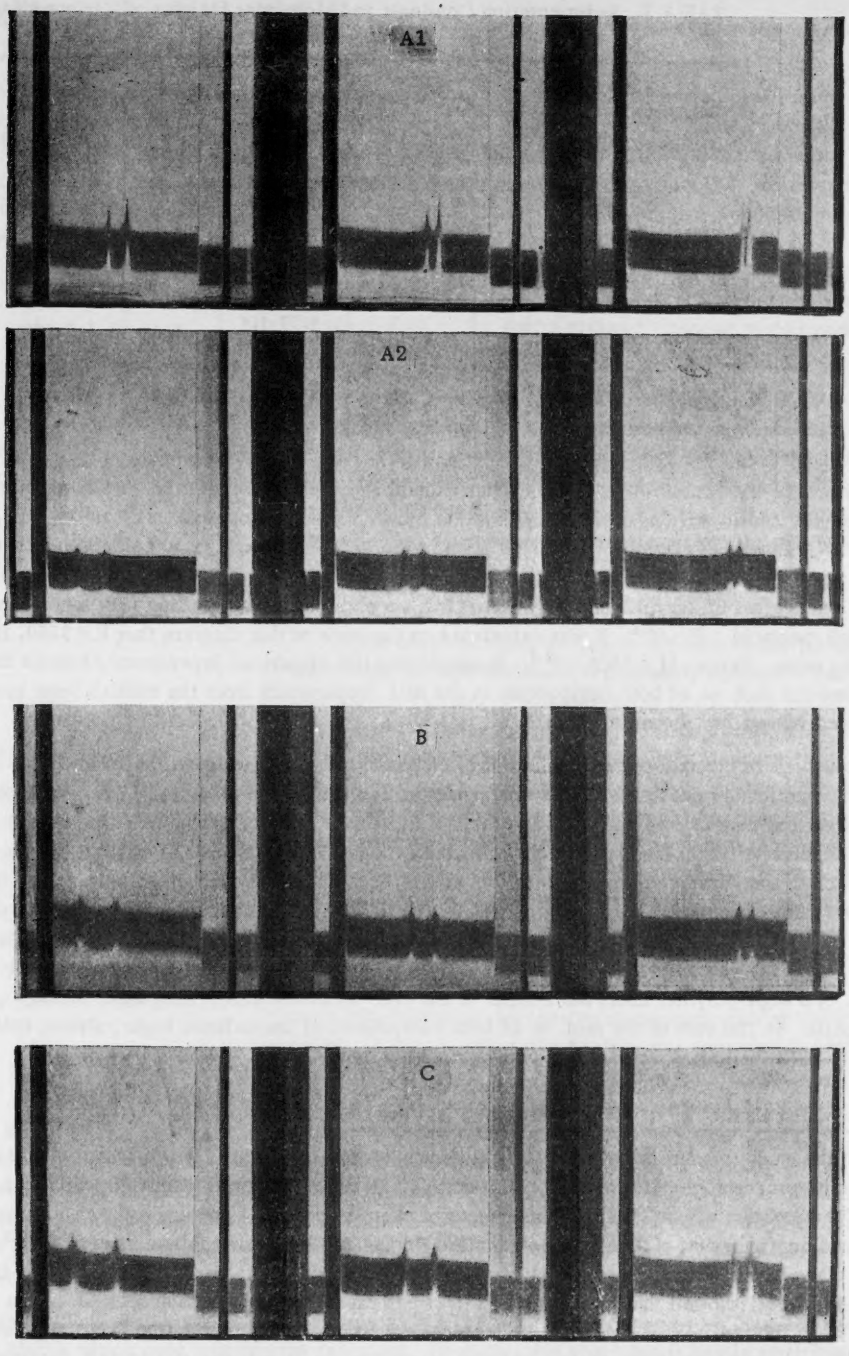

Fig. 1. Sedimentation diagrams of preparations of high-polymer RNAs from cells of various origin. 50,740 vol/min 20°, peak movement from right to left, photo interval 16 min, solvent—phosphate buffer, pH 7.3 micron 0.1. A 1) RNA from E. coli, concentration 3.6 mg/ml; A 2) another RNA preparation from E. coli, 2 mg/ml; B) from rabbit's bone marrow, 2.5 mg/ml; C) RNA from pea shoots, 2.5 mg/ml.

TABLE 1. Sedimentation Constants and Molecular Weights of Various RNAs

RNA source	$S_{20,w}$ at C_{RNA} = 2.5 mg/ml Swedb. unit)	$S^0_{20,w}$ at $C \to 0$ (swedb. unit)	Molec. w.	Correlations of molec. w. of the components	Total of molec. w. of the components
TMV	20,0	31,0	$2{,}1 \cdot 10^6$	—	$2{,}1 \cdot 10^6$
E. coli	15,2	23,0	$1{,}12 \cdot 10^6$	2:1	$1{,}7 \cdot 10^6$
	12,2	16,7	$0{,}56 \cdot 10^6$		
Rabbit	16,1	24,5	$1{,}27 \cdot 10^6$	3:1	$1{,}7 \cdot 10^6$
	11,5	14,5	$0{,}43 \cdot 10^6$		
Pea	16,5	25,1	$1{,}34 \cdot 10^6$	3,5:1	$1{,}7 \cdot 10^6$
	11,0	13,9	$0{,}39 \cdot 10^6$		

components, $1.12 \cdot 10^6$ and $0.56 \cdot 10^6$ [17]. Under the above indicated conditions, we determined experimentally the $S_{20,w}$ of the known weights of these RNAs which proved to be respectively: 31.0 for RNA of the TMV; 23.0 for the larger RNA component of E. coli; 16.7 for the smaller RNA component of E. coli (in Swedberg units). Placing on the diagram the logarithm magnitudes of the $S_{20,w}$ values ($\log S^0_{20,w}$) against the logarithm magnitudes of the known mol. w. values of the respective RNAs ($\log M$), we obtained a straight line with a gradient $\log S / \log M = 2.1$, which means $M = K \cdot S^{2.1}$. It was calculated on the basis of this diagram that $K = 1550$, if $S_{20,w}$ is expressed in Swedberg units. Hence, $M = 1550 \cdot S^{2.1}$. In employing this empirical dependence between the mol. w. and $S_{20,w}$, we computed the mol. w. of both components in the RNA preparations from the rabbit's bone marrow and pea shoots. All computed values are shown in Table 1.

On the basis of the sedimentation data and computation of mol. weights, the following rules can be outlined for high-polymer RNAs of cells of various types (bacterial, animal, and plantar). 1) In complete conformity with the data of various authors who had investigated RNA of various types of cells, the cellular high-polymer (ribosomal) RNAs of most diverse origin are bipartite as far as their mol. w. is concerned, in contrast to the monopartite viral RNA; 2) Each of the components of the high-polymer RNA is apparently sufficiently dispersive in regard to mol. w. 3) The small component of the high-polymer RNA cells is 2-3 fold smaller than the large RNA component in regard to the mol. w. 4) The mol. w. of similar components of the high-polymer RNA may differ somewhat in cells of various origin. 5) If we compare the RNA from various types of cells, we find that the larger the mol. w. of the large component of a high-polymer RNA, the smaller is the mol. w. of the small component of the high-polymer RNA of the same cells. 6) The sum of the mol. w. of both components of the cellular high-polymer RNA is identical for various types of cells, bacterial, animal and plantar, and comprises $\approx 1.7 \cdot 10^6$.

The "Temperature Effect" of High-Polymer RNAs

Upon the study of solutions of the native (infectious) RNA from TMV, it was shown that, as a result of heating the solution, the macromolecules of viral RNA undergo a definite structural transition which can be elicited in the form of a "temperature effect" [6, 7]. This structural change consists of a staggered transition of a coiled (compact) and regulated configuration of RNA into an uncoiled (loose) and irregular configuration of the chain, i.e., a "melting" takes place of the secondary and tertiary structures of the high-polymer viral RNA [6, 7]. Investigation of the "temperature effect" showed that as a result of this structural transition, the high mol. w. of the infectious viral RNA does not change substantially, i.e., that this RNA does not become dissociated after heating (upon disruption of hydrogen bonds and uncoiling of the chains) and that, consequently, it behaves as a united, continuous polynucleotide chain [7]. The emergence of breaks in the chain which take place during a spontaneous inactivation of viral RNA results in a condition where such an RNA, though retaining the high mol. w. of its macroparticles at room temperature, ceases to reveal the sp. vis. rise when the solution is heated, because during the heating process the intial inactive macromolecule dissociates into shorter chains [6]. Thus the presence of a repeated sp. vis. rise when the RNA solution is heated (temperature effect) proved to be a good criterion in ascertaining the native character, wholeness, and continuity of the polynucleotide chain in the macromolecules of high-polymer viral RNA [6].

In the present work the study of the effect of heating on the state of RNA macromolecules was expanded to various high-polymer RNAs of cellular origin.

The curves in Fig. 2 show the temperature dependence of reduced viscosity for various preparations of high-polymer RNAs in a phosphate buffer, pH 7.3 micron 0.1, in the presence of 0.01 M versen. These curves show that all investigated high-polymer RNAs—viral (Fig. 2, A), bacterial (Fig. 2, B), plantar (Fig. 2, C), and animal (Fig. 2, D)—present an analogous staggered rise of viscosity when the solution is heated, i.e., they manifest the temperature effect. It is important to point out that these viscosity changes depending on the temperature proved to be reversible in analogy with the infectious viral RNA [6, 7], so that as a result of cooling of the heated solution the viscosity magnitudes reverted to their initial levels. Finally, the presence of even relatively large concentrations of versen had no effect on the magnitude of the rise of reduced viscosity as compared with the tests carried out in a phosphate buffer without versen.

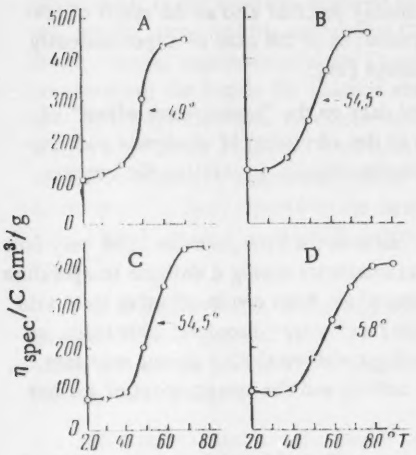

Fig. 2. Temperature dependence of reduced viscosity of the preparations of high-polymer RNAs of various origin. Solvent—phosphate buffer, pH 7.3, micron 0.1 in the presence of 0.01 M versen. A) RNA from TMV, concentration 1.6 mg/ml; B) RNA from E. coli, 2.3 mg/ml; C) RNA from pea shoots, 2.5 mg per ml; D) RNA from rabbit's bone marrow, 2.5 mg/ml.

Special studies of temperature dependence of UF-absorption and the carrying out of sedimentation experiments at increased temperatures on preparations of high-polymer RNAs from E. coli showed that here, in analogy with viral RNA, the rise of viscosity after heating is accompanied by a corresponding reduction of sedimentation rate and disruption of hydrogen bonds between the bases, i.e., it directly reflects the uncoiling of RNA chains and "melting" of the secondary and tertiary structures.

The obtained data, as well as the data of our preliminary work together with Mil'man [19], directly contradict the concepts that high-molecular (ribosomal) RNAs of the cells do not represent continuous polynucleic chains, but consist of relatively short chains—sub-units—connected via non-covalent bonds [14, 25-27]. Certain conjectures have been made that the size of these sub-units corresponds to the size of particles of soluble RNA [26, 27]. These assumptions are based on the premise that, as a result of heating of the solutions of high-polymer RNAs, certain authors obtained a sharp irreversible reduction of sp. vis. and $S_{20,w}$ which attests, in their opinion, to the dissociation of the initial macromolecules into much smaller chains, or "sub-units" [25-27]. However, in our present work we were unable to find such reduction in sp. vis. following the heating of solutions of high-polymer RNAs. On the contrary, the heating was accompanied by a sharp rise in specific viscosity at a corresponding temperature, and these changes were of a reversible nature. The presence of a considerable rise of specific viscosity of high-polymer RNA upon heating, as shown by our experiments, does not conform to the ideas of a far-advanced dissociation of macromolecules and speak, apparently, in analogy with viral infectious RNA [6, 7], in favor of uncoiling of fairly long chains. The reversibility of viscosity changes speaks in favor of the concept that, as a result of the uncoiling of the chain and disruption of hydrogen bonds upon heating, no dissociation of the initial macromolecules and their separation into shorter chains or sub-units takes place. Hence, in these experiments the high-polymer RNAs while manifesting the temperature effect still act as long, continuous polynucleotide chains.

An objection can be made that in the given experiments brief heating is insufficient to induce a dissociation of some non-covalent chains which hold together the sub-units. However, this objection is hardly well-grounded. Of the non-covalent bonds capable of holding together the sub-units more or less firmly in a macromolecule, only hydrogen bonds between nitrous bases of various "sub-units" can be taken into account, or bonds via metallic complexes (through the cations of bi- or multi-valent metals). The absence of an immediate dissociation after heating high-polymer RNAs up to 70-90° (the disruption temperature of hydrogen bonds between fairly long polynucleotide chains), as well as the reaching a maximal hyperchromic effect of the polymeric RNA at the temperature of uncoiling which attest to the absence in this case of a noticeable quantity of non-disrupted hydrogen bonds—all this renders very improbable the possibility of tightening separate sub-units into a single macromolecule via hydrogen bonds

between the bases On the other hand, the absence,even at its relatively large concentrations,of the versen effect, on the viscosity, sedimentation and mol. w. of RNA at room temperature, especially on the magnitude of the increase of viscosity after heating, speaks also against the second possibility—the fixation of the sub-units by means of metals.

Hence, high-polymer RNA most likely represents unified, continuous polynucleotide chains consisting of nucleotide radicals united by covalent (phosphodiester) bonds (1300-1900 nucleotides for the smaller component and 3700-4500 nucleotides for the larger component, respectively). The "dissociation" of macromolecules by a high-molecular RNA upon heating, especially after prolonged (about an hour) heating [26, 27], which has been reported by some authors [14, 25-27], can presumably be regarded as a thermal degradation of a high-polymer RNA, i.e., as disintegration of a previously continuous polynucleotide chain, as the result of disruption of a certain number of covalent inter-nucleotide bonds. Sometimes such a "dissociation" is presumably possible also as the result of previous injuries to the RNA chain which were not manifested at room temperature, as in the case of a spontaneously inactivated viral RNA [6] or rat RNA after a certain period of prolonged storage [19].

Thus, the first deduction which can be made on the basis of submitted data on the "temperature effect" of high-polymer cellular RNAs of animal, plantar, or bacterial origin consists of the admission of wholeness and continuity of the polynucleotide chains in the native RNA preparations and, correspondingly, in refuting the concept that they represent aggregates of a large number of low polymer sub-units.

The next deduction concerns the configuration of the polynucleotide chain in the RNA particle. The very fact of elicitation of the staggered type of changes of their physicochemical characteristics during a definite temperature interval conveys the impression that under given conditions a transition takes place from one qualitative state into another, a type of phasic transition, or a transition which can be characterized as "order—disorder." This leads us directly to the conclusion that high-polymer cellular RNA in solution in analogy with viral RNA do not represent irregularly coiled chains, but are characterized by a definite order in their coiling and the arrangement of various sections, i.e., the presence of secondary and tertiary structures.

Finally, it should be pointed out that the "temperature effect" which is manifested by high-polymer RNAs of various origin represents a repetition of an analogous phenomenon in viral RNA in solution. This fact leads us to another conclusion, namely, that the macromolecular configuration of the chain of cellular RNAs and the very nature of the structural regularity in them is principally analogous to those of the native viral RNA in solution. This is an additional and, perhaps, one of the most substantial reasons in favor of the concept that our basic ideas of the secondary and tertiary structures, formulated mainly on the basis of study of the infectious RNA of the TMV [11], are also fully applicable to the high-polymer RNA of most diverse origin.

Comparative Characteristics of Molecular "Melting" and the Similarity of the Macrostructure of High-Polymer RNAs

A comparison of the temperature profiles on the rise of reduced viscosity (Fig. 2) reflecting the "melting" profiles of secondary and tertiary structures of high-polymer RNAs in solution under given conditions indicates that, despite the general similarity in the form of curves (width of transition, magnitude of rise, etc.), one intersting difference can be observed. The difference is in the magnitudes of temperatures at which the transition takes place.

In order to correctly evaluate the transition temperature the melting temperature (M.P.) of the secondary and tertiary structure, one can employ the Doty method who accepted as mean M. P. of the secondary RNA structure the temperature which corresponded to the middle of the curve of temperature dependence of the UF-absorption of RNA solutions [28]. In our case, as the mean M.P. of the RNA structure we can take the temperature which corresponds to the middle of the curve of the temperature dependence of reduced viscosity (Fig. 2).

The curves of temperature dependence of viscosity in Fig. 2 show that RNAs of various origin have different mean temperatures of molecular melting—from 49° in RNA or TMV to 58° in rabbit's RNA.

Various RNA preparations from the same experimental object show remarkable characteristics of the mean M.P. magnitudes (within the ± 0.5 range). It is interesting that in experiments with versen the character of the transition curve and the transition temperature do not depend on the RNA concentration in the solution (within certain limits), in contrast to our previous deductions relating to viral RNA in solutions without versen [7]. It may be assumed that the shift of curves of "melting" to the high-temperature zone and the narrowing of the temperature

interval of transition, observed earlier on viral RNAs when the RNA concentration in solution had been increased [7], is due not to the simple electrolytic effect, but to the specific action of bivalent metals present in the RNA preparations; the increase of RNA concentration indicated also an increased concentration of bivalent metals present in RNA preparations, and bivalent metals as is known [29] stabilize the RNA structure and raise the M. P. of spirals. Therefore, the use of versen which removed the effect of metals represented an important step which ensured in the present work the independence of "melting" curves from varying admixtures of metals and, consequently, also the independence of these curves from RNA concentrations, thus enabling us to obtain a good reproduction from one preparation to the other.

Since the differences in the mean temperature of the structural transition of investigated RNA preparations could not be explained by accidental variations of the preparations, or by the concentration of the polyelectrolyte (RNA) itself in the solution, it would be logical to regard them as certain structural differences of the RNA of various origin. Doty and his co-workers [28] showed earlier that the mean M. P. of DNA spirals, elicited from the curves of the thermal dependence of the UF-absorption of DNA solutions, is in direct correlation with the nucleotide DNA composition: the higher the guanine and cytosine content (G + C) in the DNA molecule, the higher is the mean M.P. of the spirals. This fact gave us the idea of ascertaining in our work whether there is a correlation between the temperature of the structural transition of high-polymer RNAs and the relative content of G + C in them. In Table 2 are shown the mean magnitudes of the RNA M.P. elicited from the curves of thermal dependence of the reduced viscosity (Fig. 2), as compared to the data in the literature on the nucleotide composition of high-polymer RNAs (for TMV [30], E. coli [31], pea [32], rabbit [33], and rat [34]). The data for RNA of a rat are derived from the previous work [19].

It is seen in Table 2 that the higher the G + C content in RNA, the higher the mean M. P. detected by the staggered rise of reduced viscosity. Correspondingly, the higher the G + C content, the higher are the temperatures which are needed for the achievement of maximal uncoiling of the chains and disruption of hydrogen bonds (see plateau area in Fig. 2).

In order to establish the nature of the detected correlation, the values of the mean RNA melting points were plotted against the values of the molar ratio of total G + C, correspondingly to each of the five various RNAs (Fig. 3). A simple linear relation was observed indicating a fairly strict correlation between the mean temperature transition values and the G + C content in RNA. This direct correlation indicates that the temperature of structural transition represents a simple linear function of a single structural factor—the nucleotide RNA composition—thus permitting, with a certain degree of probability, the exclusion of the effect of any other independent factors not connected with the nucleotide composition.

Theoretically, the temperature of structural transition, i.e., the strength of secondary and tertiary RNA structures depends on the following independent factors: the average nucleotide composition of the spiral segments—the more G - C pairs (with three hydrogen bonds) as compared with the number of A - U pairs (with two hydrogen bonds) the stronger are the spiral segments [9, 28, 35]; the average length of the spiral segments: the longer the spiral segments, the more stable they are [9, 35]; the average number of non-paired bases ("defects") within the spiral segments: the greater their number, the less stable is the spiral [9, 35, 36]; the total strength and stability of the mutual compactness of the spirals, i.e., the strength of the tertiary structure as such [11]. Had all these four independent structural factors (or more than one of them) varied in different RNAs, we would not of course, be able to observe the simple linear dependence of the temperature of structural transition from any of these factors. Hence, since we do observe experimentally the dependence from one of these factors—the nucleotide composition—and since this dependence has the character of a simple linear dependence unaffected by any other influences, we may assume that all other enumerated factors do not vary to any considerable extent in RNAs of various origin. In other words, the obtained data can apparently be interpreted as a certain proof in favor of the common nature of the basic principal features of the secondary and tertiary structures in solution in high polymer RNAs of most diverse origin: viral, bacterial, plantar, and animal. Not only are the two-string character of the spiral segments of RNA and the specific character of pairing of bases (G-C and A-U) common to all RNAs, but also apparently the average length of the spiral segments and the character of compactness and distribution of the spiral segments in relation to each other, as well as the average number of non-paired bases (loops), are identical in all RNAs. In any case, in the characterization of the macromolecular structure of high-polymer RNAs in solutions, we can apparently employ the same principles of primary, secondary, and tertiary structures which have been formulated for the native (infectious) viral RNA [11].

TABLE 2. Temperature Values of the Structural Transition and Indices of the Nucleotide Composition of High-Polymer RNAs

RNA source	Mean transition temp. melting point	Plateau area temperature maximum	$\frac{G+C}{A+U}$	G + C, in percentages
TMV	49°	60—70°	0,78	44
E. coli	54,5°	70—80°	1,16	54
Pea	54,5°	70—80°	1,20	55
Rat	58°	80—90°	1,52	60
Rabbit	58°	80—90°	1,54	61

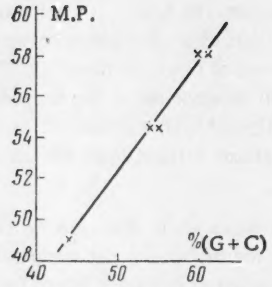

Fig. 3. Diagram of the dependence of the mean temperature of "melting" (M.P.) on the nucleotide composition of high-polymer RNAs of various origin (TMV, E. coli, pea, rabbit, rat). Abscissa—magnitudes of the molar content of G + C in percentages of the total sum of bases.

SUMMARY

A comparative study has been carried out of high-polymer RNAs isolated from the cells of E. coli, pea shoots, rabbit's bone marrow, and from the tobacco mosaic virus.

The study of sedimentation in an ultracentrifuge showed that all cellular high-polymer RNAs, in contrast to viral RNA, are bi-component as to their molecular weight, the small component being 2 to 3.5 times (depending on the type of cells) smaller than the large component. The molecular weights of similar components and the magnitude ratio of the molecular weights of the two components of cellular high-polymer RNAs differ somewhat in the cells of various origin, but at the same time the sum of the molecular weights of both components in all three investigated types of cells comprises an identical figure corresponding to the molecular weight $1.7 \cdot 10^6$.

Upon heating the high-polymer RNAs in solution they develop a staggered rise of specific gravity in a definite thermal interval ("temperature effect"). An analogous behavior of high-polymer RNAs upon heating speaks in favor of a similar nature of structural transition in RNAs of various origin and, consequently, of the analogous character of their configurational order (secondary and tertiary structures).

The repeated increase of specific viscosity, upon the uncoiling of RNA chains under the effect of heating, permits us to assume that all investigated high-polymer RNAs represent continuous polynucleotides, and do not consist of short sub-units held together by secondary bonds.

The mean temperature of the structural transition (the temperature of molecular "melting") of high-polymer RNAs in solution differs somewhat in various RNAs and depends on their nucleotide composition. A direct linear dependence has been elicited between the temperature of "melting" and the relative content of guanine and cytosine in RNA—a phenomenon which permits us to regard the nucleotide composition as the sole factor affecting the transition temperature.

A conclusion has been arrived at of the principal identity of the basic characteristics of secondary and tertiary structures of high-polymers RNAs of various origin in solution.

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AGAR GEL ELECTROPHORETIC INVESTIGATION OF SOLUBLE CYTOPLASMIC AND NUCLEAR PROTEINS OF RAT LIVER AND TRANSPLANTABLE RAT HEPATOMA

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Proteins of normal and tumor tissue have been the objects of numerous investigations. Interest in this problem is completely understandable, if we consider the important role which they play in the determination of structural and metabolic differences of tumor cells. However, in spite of the many investigations in this area, our knowledge of the relative quantitative and qualitative differences between proteins of normal and tumor cells is still very incomplete; even the existence of such differences is argued.

Experimental data on the proteins of tumor tissue have been examined in a number of review articles [1-6]. Sufficiently reliable data does not now exist on the presence of proteins characteristic only for tumor cells and qualitatively different from the normal cells. On the other hand, investigation of protein fractions in tumors by means of electrophoretic [7-10] sedimentation [11] or immunological [12-14] methods have shown a simplification of the protein profile and the absence of certain protein fractions characteristic for normal homologous tissue. These data are an essential argument in favor of the hypothesis of J. and E. Miller [15-16] that the reason for malignization is the disappearance of certain intracellular proteins. Almost all the investigations indicated above were carried out with extracts from whole homogenates of normal and tumor tissues. It would be especially interesting to study the protein fractions from separate intracellular structures. Such investigations have been carried out by a number of authors [5]; however, electrophoretic analysis has not been used extensively enough.

In a study of tumor biochemistry, the most suitable material for investigation is the liver, as well as primary and transplantable tumors of the liver, since the changes found can be compared with the original homologous tissue. A number of authors have separated soluble proteins of the liver electrophoretically by means of free electrophoresis [7, 9, 17-19], paper electrophoresis [20, 21], or an agar gel [22-25]. All these authors have worked with extracts of intracellular structures. Lamirande et al., [26] fractionated by means of free electrophoresis soluble proteins from separate intracellular structures of normal rat liver and rats receiving 4-dimethylaminoazobenzene in their food.

Considering the high fractionating ability of electrophoresis in agar gel [22, 23], in which quantitative determinations are quite accurate, and considering the lack of investigations of soluble proteins from intracellular structures by these methods, we determined to investigate the electrophoretic mobility in agar gel of soluble proteins from isolated nuclei and cytoplasm of rat liver, as well as from forms of transplantable hepatoma.

METHODS

The experiments were carried out on white rats weighing 150-200 g. Liver tissue and transplantable solid hepatoma of rats, primarily induced by 4-dimethylaminoazobenzene * were the experimental material. Pieces of tumor tissue or cellular suspensions were transplanted intramuscularly into the posterior extremity. Since rapidly growing tumor quickly necrotizes, we employed dense tumor nodes approximately 1 cm in diameter. Histologically the tumor presented a picture of typical hepatoma (Fig. 1). Eighteen hours before the animals were killed no food was withheld. A comparative investigation was also carried out with protein extracts from whole liver of rat embryos 2 cm long. The chest cages of the animals under ether narcosis were opened and the liver then perfused in situ through the heart with cold solution (4°) of 0.85% NaCl (approximately 300 ml) and 0.25 M sucrose, containing

*The tumor tissue was kindly furnished to us by Prof. A. Grafi, Institute of Biology and Medicine of the German Academy of Science, Berlin.

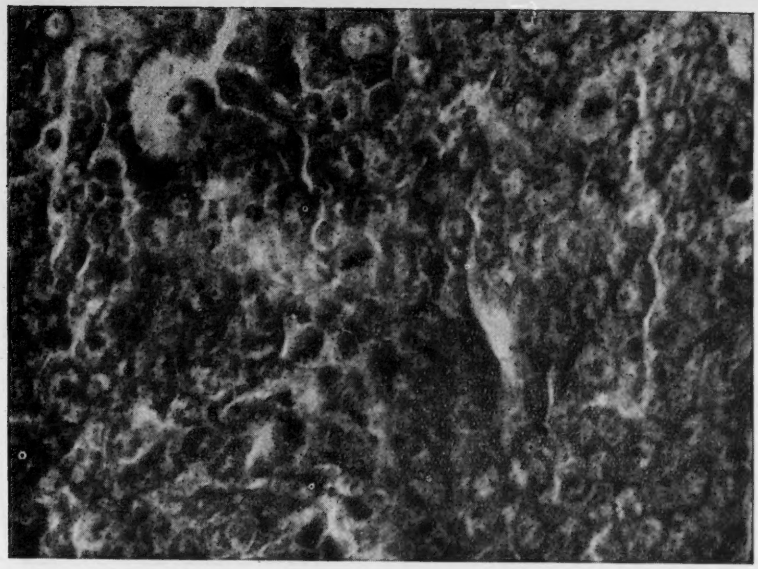

Fig. 1. Structure of rat transplantable hepatoma. Stained with methyl green pyronine. Objective 45, ocular, 10.

0.0018 M $CaCl_2$ (approximately 100 ml). The liver was perfused for approximately 5 min; the organ was thus washed free from blood. All further procedures were carried out in the cold.

A weighed portion of the tissue after careful pulverization was homogenized in a glass Potter-Elvehjem homogenizer with equal quantities (weight-volume) of 0.44 M sucrose containing 0.0036 M $CaCl_2$. After centrifugation for 10 min at 2400 g the supernatant liquid was removed (later, referred to as the cytoplasmic fraction), stirred carefully with n-butanol (1 : 4) for 30 min [27] and centrifuged for 15 min at 2400 g. The extract was drawn off by suction, treated three times with equal volumes of ether [19] and again centrifuged 10 min at 2400 g. As a result a transparent pale-yellow extract was obtained, consisting of 0.8-1.2% cytoplasmic proteins.

From the other portion of the liver tissue a 10% homogenate was prepared in 0.25 M sucrose, containing 0.0018 M $CaCl_2$. From the homogenate the nuclear fraction was isolated according to the method of Hogeboom et al. [28], which we applied as follows: the homogenate was taken up in an equal volume of 0.34 M sucrose, containing 0.0018 M $CaCl_2$, and centrifuged at 600 g for 5-10 min. The supernatant liquid was poured off, and the precipitate resuspended by means of a freely moving pestle in 0.25 M sucrose, containing 0.00018 M $CaCl_2$. The nuclear suspension obtained was sweetened by means of a release pipette with an equal volume of 0.34 M sucrose containing 0.00018 M $CaCl_2$ and centrifuged at 600 g for 5-10 min. The supernatant liquid was poured off and the precipitate (nuclear fraction) dried, transferred to a glass homogenizer and homogenized in a half quantity (by weight) of borate buffer, pH 8.6 μ 0.16. After maceration of the nuclei for 48 hr at 4° an extract was obtained, containing soluble protein, which was further treated by the following two methods: 1) Immediately after centrifugation for 15 min at 5000 g the supernatant liquid was poured off, extracted three times with ether, again centrifuged 10 min at 2400 g. A strongly opalescent extract was obtained, tinted red with hemoglobin from the erythrocytes mixed with the nuclei, having a protein content of 1-1.3%. 2) To the macerate was added n-butanol in a ratio of 1 : 4 and after homogenization and standing for 30 min at 4° it was centrifuged for 15 min at 5000 g. The separated extract was treated in this way three times and again centrifuged for 10 min at 2400 g. As a result, a light opalescent yellow extract was obtained, free from hemoglobin, containing from 0.3-0.4% protein.

Only the cytoplasmic fraction of the tumor tissue was investigated because of its limited quantity. A whole 10% homogenate in 0.15% M NaCl was obtained from rat embryos immediately after its maceration for 48 hr at 4° and treatment with ether, similar to the first extraction method of the nuclear fraction.

Microscopic investigation with Romanovskii-Giemsa stained smears from the cytoplasmic fractions did not show any nuclei or residues of undestroyed cells. Reaction of this fraction with DNA and diphenylamine [29] was negative. The purity of the nuclear fractions was controlled by stained smears (Romanovskii-Giemsa).

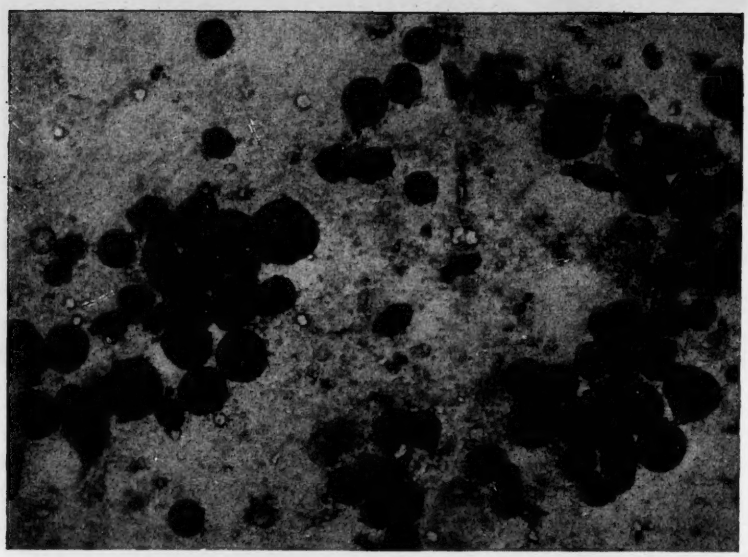

Fig. 2. Isolated nuclei of normal liver cells. Stained with Romanovskii-Giemsa. Objective, 45, ocular 10.

As may be seen from Fig. 2, this method permitted us to obtain well-preserved nuclei, free from protoplasm. The percentage of undestroyed cells varied from 1.25 to 1.5%. According to data in the literature [28] the nuclear fraction obtained by this method contains approximately 0.5% of mitochondria of whole homogenate.

The protein content in the extracts was determined by Lowry's method; hemoglobin by Ardrey and Stork. The protein extracts were subjected to zonal electrophoresis in agar medium according to the method of Grabar [30] and Giri [31] with Ilkov and Nikolov's modification [32]. Electrophoresis was carried out in borate buffer, pH 8.6 μ 0.04. Electrophoresis was continued for 3-3.5 hr at a potential gradient of 5 v/cm. The electrophoretograms were stained with amide black 10 B and measured photometrically in a registering extinctionometer (Model 11 Zeiss).

RESULTS OF THE INVESTIGATION

Quantitative distribution of the proteins in various fractions is indicated in the table.

M_a – arithmetical mean; m_a – mean square error M_a; n – number of experimental animals.

Fraction	Cytoplasm of normal liver, $M_a \pm ma$ (n = 24)	Cytoplasm of transplantable hepatoma $M_a \pm ma$ (n = 8)	Nuclei of normal liver $M_a \pm ma$ (n = 12)	Fraction	Cytoplasm of normal liver, $M_a \pm ma$ (n = 24)	Cytoplasm of transplantable hepatoma, $M_a \pm ma$ (n = 8)	Nuclei of normal liver, $M_a \pm ma$ (n = 12)
A	1,6±0,2	0,6±0,2	1,9±0,3	G	7,7±0,6	6,6±0,1	6,4±0,7
B	1,7±0,2	1,5±0,2	7,3±0,5	H	11,6±0,3	7,3±0,1	9,2±0,6
C	2,1±0,3	10,4±0,3		I	11,4±0,2	7,2±0,4	8,5±0,6
D	5,2±0,4		7,4±0,1	J	20,8±0,6	16,3±0,4	10,0±0,1
E	8,5±0,3	34,1±0,5	8,0±0,4	K	4,6±0,3	9,1±0,1	6,1±0,2
F	9,4±0,6		26,9±0,6	L	2,9±0,2	2,3±0,2	2,7±0,1
				M	12,5±0,4	4,6±6,2	5,6±0,1

Cytoplasm extracts of normal rat liver contained 18-22% cytoplasmic protein and on electrophoresis this was distributed in 13 well-defined fractions (Fig. 3, a). Some of these fractions were nonhomogeneous. Fractions A and C had higher mobility than the corresponding fractions of serum albumin (compare Fig. 4a and 4d). Proteins with the

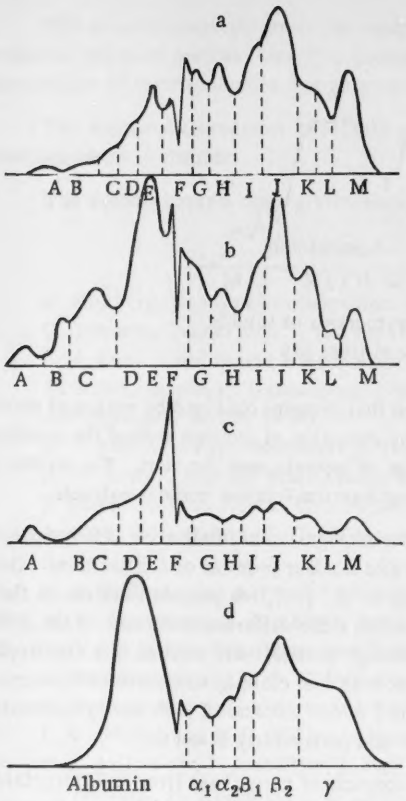

Fig. 3. Densitometric curves of electrophoretograms of cytoplasmic liver proteins (a), transplantable rat hepatoma (b), nulcear proteins of liver (c) and rat blood serum (d).

mobility of serum albumin were comparatively small (18-23%). The well-defined fraction M, having the least mobility corresponded to the slowest-moving of all, serum γ-globulin. Fraction J, was particularly outstanding quantitatively, since it contained 21% of the soluble liver proteins.

Extracts from liver nuclei contained approximately 10% of the total protein of the nucleus when extracted with solvents without n-butanol and approximately 4% on extraction with n-butanol. In spite of this difference, the electrophoretograms of both extracts were similar; extracts obtained without n-butanol differed only in that they contained a large hemoglobin fraction, occurring in the region of fraction I and J. Correcting for the hemoglobin (16-20% of the total protein) the electrophoretograms obtained from both extracts could be compared. Supplementary experiments with extracts obtained by this method from erythrocytes (after maceration and without n-butanol, Fig. 4) showed that hemoglobin contained, approximately 90% of the extractable protein. Consequently, contamination of nuclear protein fractions with other proteins from erythrocytes was insignificant and could not significantly affect the results. Electrophoresis of nuclear extracts (Fig. 3, c) revealed at least 12 protein fractions. Fractions B and C in these experiments were clearly non-homogenous, while fractions I and J were poorly separated. In comparison with the cytoplasmic extracts, proteins with the mobility of serum albumins were present in the nuclear extracts in large quantities, 42% on an average; this was particularly noticeable in the starting fraction F. Content of prealbumin fractions in nuclear extracts was higher, and fraction M, having minimal mobility, was relatively decreased.

Electrophoretic investigation of the cytoplasmic extracts of transplantable hepatoma (Fig. 3, b) showed almost the same number of fractions as in the corresponding extracts of normal rat liver. However, the hepatoma fractions appeared to be more homogenous, and fractions D, E, and F were poorly delimited from each other. The prealbumin fractions A and B were more mobile than the corresponding fractions of normal liver extracts. The low content of fraction M in extracts of hepatoma is noticeable, because the percentage content of fractions with mobility of serum albumin (fractions D, E, F and especially C) were thereby increased. The total content of these fractions was on the average 44.3% of the protein extracts.

On the electrophoretograms of the embryonal liver extracts (Fig. 4) a massive band of hemoglobin was found, which masked the protein fractions H, I, J, K. In spite of this, however, it is evident that the extracts from embryonal liver were electrophoretically more homogenous, contained more proteins with the mobility of serum albumin (pronounced peak of fraction C), fraction M in these extracts was definitely decreased, and the prealbumin fractions had more mobility than the corresponding fractions of normal liver. In general, the electrophoretograms of embryonal liver were extremely similar to the electrophoretograms of hepatoma.

DISCUSSION OF THE RESULTS

Our electrophoretograms of soluble cytoplasmic proteins of normal rat livers are similar to electrophoretograms obtained by a small number of authors, who also separated liver proteins by means of zonal electrophoresis in agar medium [22-25]: one or several prealbumin fractions were found on the electrophoretograms, a comparatively small number of proteins with mobilities of serum albumin, and approximately the same number of fractions. Differences between the data of the authors mentioned above and our data probably depend on differences in technique of electrophoresis, methods of extraction, species differences in experimental animals and the fact that in some of these papers non-perfused liver tissue was investigated. At the same time it is necessary to note that all these studies mentioned above were carried out in alkaline homogenates of liver, extracts of which, in addition to the cytoplasmic

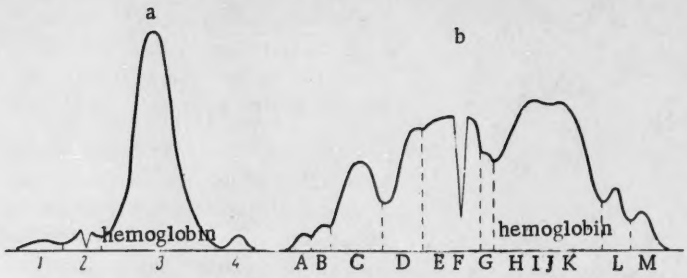

Fig. 4. Densitometric curve of the electrophoretograms of soluble proteins of rat erythrocytes (a) and rat embryonal liver (b).

proteins obviously contain nuclear proteins. The impression is created that proteins obtained by means of maceration of extracts [22, 23] are partially denatured, which is evidenced by deviation of proteins around the starting strip. Extracts, obtained without maceration [19, 27], contain less percentage of protein near the start. The partial denaturation on extraction could explain the increased content of starting fraction F in our nuclear extracts.

In spite of the different methods of protein extraction, there were no significant qualitative differences observed in the electrophoretograms obtained from soluble cytoplasmic and nuclear proteins of normal liver. On free electrophoresis of the extracts obtained after maceration by Lamirande et al. [26], five protein fractions in the supernatant liquid and only three in the nuclear extracts were found. Probably, these differences depend on the differences in the extraction methods or techniques of electrophoresis. Although qualitatively similar, the electrophoretograms which we obtained from soluble cytoplasmic and nuclear proteins had clear quantitative differences: a decreased relative content in the nuclei of the slowly moving fractions J and M compared with the cytoplasmic extracts, and an increased percentage content of prealbumin fractions A and particularly B and C.

The differences in the electrophoretograms of the cytoplasmic extracts of normal rat liver and transplantable hepatoma are very interesting. These differences are either qualitative: increased mobility of the prealbumin fractions A and B, or particularly quantitative : significant decrease in the relative content of the slower moving fraction M and the marked increase in content of fractions D, E, and F, and C in particular. The soluble proteins of cell cytoplasm of hepatoma contain more proteins with the mobility of serum albumin and fewer with the mobility of serum γ-globulin. Probably, fraction M in our experiments corresponds to fraction 6 in the experiments of Lamirande et al. [26], and fraction h in the experiments of Sorof and Cohen [7]. As is known, this fraction is selectively bound with cancerogenic azo dyes [8, 9] and is greatly decreased in various transplantable tumors, as well as in primary induced tumors of liver [7, 8, 10, 26]. In our experiments this was verified with respect to the transplantable hepatoma we investigated. In addition to this, in our experiments there was a clearly expressed increased in the relative content of proteins with the mobility of serum albumin fractions C, D, E, and F. These fractions, probably, correspond with the fractions A and H, an increased content of which was established by Sorof and Cohen in liver tumors induced with azo dyes [7]. Our data indicate that this increase is characteristic for all fractions and not only for some of them.

It must be emphasized that electrophoretograms of soluble cytoplasmic proteins of hepatoma and embryonal liver, in spite of the differences of extraction methods, are entirely similar; and in this and the other case a decrease in fraction M, increase in fractions C, D, E, and F, and increase in the mobility of the prealbumin fractions are noted. These data are evidence that the changes studied here are not specific for tumor tissue and fortify the hypothesis stated by Hoffman and Schechtman [9] that the electrophoretic picture is characteristic for all rapidly growing tissue.

SUMMARY

A method is described for the extraction of cytoplasmic and nuclear proteins of liver, in which the extracted proteins are well-fractionated by electrophoresis on agar gel.

The fractional composition of the cytoplasmic and nuclear protein extracts is not identical; they differ primarily in relation to the content of certain fractions.

The electrophoretograms of the cytoplasmic protein extracts of transplantable hepatoma differ from the electrophoretograms of normal liver by a decrease in the fractions with the least mobility, increase in the fractions with the mobility of serum albumin and greater mobility of the prealbumin fractions.

The electrophoretograms of soluble proteins of liver of rat embryos and soluble cytoplasmic proteins of hepatoma are entirely similar.

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THE ENZYMATIC ACTIVITY OF SOME SNAKE VENOMS*

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The study of the composition and structure of nucleic acids is inseparably related to the study of the metabolic enzymes of nucleic aicds. A number of authors [1-3] note that snake poison is a source of enzymes, and specifically, of nucleic acid metabolic enzymes. Since, up until the present, venoms of snakes inhabiting America and Africa had been described, we were interested in investigating the enzymatic activity of snake venoms found in the Soviet Union, and in their possible use as sources of nucleic acid metabolic enzymes.

In this communication we present data on the comparative characteristics of various snake venoms as regards their phosphodiesterase and 5'-nucleotidase (phosphomonoesterase) activities.

METHODS

We used the venoms** of four types of snakes which are common, mainly, in the territories of Central Asia; the viper Vipera lebetina, the adder Echis carinatus, the cobra Naja oxiana and the South American rattlesnake.

The venom was dried at room temperature in a $CaCl_2$-containing desiccator, and then subjected to acetone fractionation by the Koerner and Sinsheimer method[1] (with very slight modification). The aqueous venom solution (16.7 mg/ml water) was kept at room temperature for one hour, and then centrifuged at 4000-5000 rev/min for 15-20 min. These centrifugation conditions were observed throughout the whole fractionation. To the first volume of supernatant fluid we added a 0.67 volume of 0.5 M ammonium-acetone buffer, pH 4.0. All further procedures were conducted at 4°. To 10 ml of the buffered venom solution (purification stage I) we added 7.25 ml of acetone which had been cooled to −20°. After 30 min, the precipitate which formed was separated by centrifugation. To the supernatant fluid (purification Stage II) we added 1.4 ml acetone, and let the solution stand for 12 hr. The mixture was centrifuged and to the supernatant fluid (Stage III) we added 1.2 ml acetone. This solution was left at 4° overnight, and then it was centrifuged. The supernatant fluid comprised Stage IV of the purification. The active sediment was dissolved in water (Stage V).

FDE activity was determined by a modified Sinsheimer method[2] with the splitting of the calcium salt of di (paranitrophenyl)−phosphoric acid, which was synthesized by Yoshida's method [4]. The incubation mixture for determining FDE activity contained 1 ml of 0.001 M calcium salt of di (paranitrophenyl) phosphoric acid, to which we added 0.05 ml of the enzyme, 2.7 ml of 0.05 M. Tris-HCl-buffer, pH 8.9 and 0.3 ml of 0.3 M magnesium acetate. The mixture was incubated at 37° for one hour. Increase in optical density was measured in the SF-4 spectrophotometer at 440 mμ. The control was a mixture of the same composition, but without the enzyme. As a unit of enzyme activity we adapted that amount of enzyme which, under the above-described incubation conditions, caused a 0.1 change in optical density at 440 mμ.

The FME activity was measured by the splitting of AMF and the accumulation of P_{inorg}. P_{inorg} was determined by a slightly modified Lowry and Lopez method [5]: in our experience, the 0.1 N sodium acetate solution, recommended by these authors, did not maintain a stable pH 4.0 upon the addition of other reactants. Therefore, instead of the 0.1 N sodium acetate, we used a 1.0 N solution. The incubation mixture consisted of 0.4 ml AMF (450 micrograms/ml), 0.05 ml of enzyme, 1.9 ml of 2 M Tris-HCl-buffer, pH 8.8 and 0.2 ml of 0.3 M

*Accepted abbreviations: FDE−phosphodiesterase; FME−phosphomonoesterase; AMF−adenosine-5-phosphate; Tris−trioxymethylamino-methane.

**The venoms were obtained from the Tashkent Zoo Venomous Snake Ecological and Toxicological Laboratory.

TABLE 1. Comparative Activities of Phosphodiesterase and 5'-Nucleotidase in the Tested Snake Venoms (The numbers presented are the average values of a number of experiments. Each determination was run with at least two parallels)

Venom type	Enzymatic activity				Activity Ratio FDE per FME
	in units of activity		in moles of substrate split		
	FME	FDE	FME · 10^7	FDE · 10^5	
Viper	2.3	2.4	3.2	10.	270
Echis	2.45	1.2	3.7	7.7	200
Cobra	2.8	2.0	4.0	9.0	220
Rattlesnake	2.4	1.8	3.7	9.0	240

magnesium acetate. The molarity of the buffer was selected so as to ensure a stable pH upon addition of the acid buffer-dissolved venom. The mixture was incubated at 37° for one hour. Extinction of the solutions was determined in an electrophotocolorimeter with a red light filter (giving maximum transmission at 610 mμ). The control was the identical mixture without the addition of enzyme.

As the unit of enzyme activity, we designated that amount of enzyme which, under the above incubation conditions, caused a 0.1 change in optical density. For establishing a comparative scale of FDE and FME activity, the activities of both enzymes were tested simultaneously with identical quantities of venom dissolved in ammonium-acetate buffer, pH 4.0. The incubation period was one hour. The comparative enzyme activity was expressed as moles of substrate split per unit of time by a standard volume of enzyme.

RESULTS OF THE EXPERIMENTS

Our experiments showed that the four snake venoms tested possess both FDE and FME activity. Data on the activity of these two enzymes, as well as their quantitative co-relationship for each venom type are presented in Table 1.

As we see from Table 1, the venoms of the different snakes varied sharply from one another in their FDE activity. The viper had the highest FDE content, while the echis showed the lowest. The tested venoms varied hardly at all, however, in their FME activity. In all cases, the FDE activity was 200-300 times that of the FME.

In performing the acetone fractionation, we were interested in the possible separation of FDE and FME, with the subsequent extraction of FDE. In this connection, we had to consider, on the one hand, the quantitative yield of FDE, and on the other, the admixture of FME impurity in the extracted FDE preparation.

FDE activity was determined at each of the purification stages—for this we withdrew 0.05 ml samples and determined all the enzymatic activities simultaneously at the completion of the fractionation. Since our fractionation procedure took three days, and the various fractions were tested simultaneously, it followed that at the time of testing the samples of stages I and II had been stored for two days, and stage III sample—for one day, whereas samples of stages IV and V were analyzed immediately.

In view of this fact, we had to determine whether the enzymatic activity of dissolved protein changes upon storage, especially in the fractions containing acetone. We therefore ran a series of tests on viper venom, as follows: from each fractionation stage we took 3 samples of 0.05 ml. One sample was analyzed immediately for FDE activity; the other two, after 24 and 48 hr, respectively.

Results of one such experiment (typical of the whole series), were as follows:

Storage time, in hr	Optical density at λ 400 mμ for fractionation stage: I	II	III	IV	V
0	0.220	0.150	0.120	0.110	0.155
24	0.200	0.150	0.135	0.130	0.180
48	0.200	0.130	0.140	0.125	0.165

As we see from these data, the enzyme activity of all three samples for each stage proved to be practically identical—we therefore concluded that our method of simultaneous FDE determination for all the purification stages was satisfactory. Our experiments showed that with cobra venom the main loss in FDE activity during acetone fractionation occurred in stage V. Two experiments, typical in method and results, show the loss (in %) of phosphodiesterase activity to be as follows:

Expt. No.	Fractionation stage I	II	III	IV	V
2	0	0	8	0	86
5	0	0	0	17	79

Experiments with echis venom yielded similar, though less exact results.

In our attempt to decrease the loss and increase the yield of FDE we tried changing the amounts of acetone added in stage III. The gradual increase of acetone from 1.2 to 2 ml did not, however, give us the desired results. When the amount of added acetone was decreased to 1 ml, precipitation did not take place.

FME activity was determined in the first and last stages i.e., in the buffered venom and in the aqueous solution of the active sediment.

Table 2 presents data on the FDE and FME contents in the final sediments of the four snake types.

Maximum FDE yield with our working methods was 8-11% for this echis, viper, and rattlesnake. The cobra's yield did not exceed 2.5%.

Moreover, of the four venoms we tested, we achieved a satisfactory separation from FME impurity only with echis venom. In the rest, the residual FME activity comprised 6-12%—in the viper, it was as high as 14.5% of the original activity.

TABLE 2. Quantities of Phosphodiesterase and Phosphomonoesterase in the Final Sediment of Acetone Fractionation. (Figures presented are average values of 20-25 experiments for each venom type)

Type of venom	Yield of FDE	Admixture of FME
	% of original activity	
Viper	10.7	14.5
Echis	8.2	< 0.3*
Cobra	2.5	12.0
Rattlesnake	10.0	6.0

*Results for FME activity of echis lie just on the limit of experimental error.

DISCUSSION OF THE RESULTS

Koerner and Sinsheimer, upon purifying FDE of FME by the acetone fractionation method, obtained, in their final sediment, 18-20% of the original FDE activity.

Our quantitative FDE yields can also be counted as satisfactory, if we consider that the above authors used lyophilized snake venoms, whereas we dealt with preparations dried in a dessicator at room temperature, which could not but affect the qualities and activity of our starting material (see ref. [6]).

We must note that measurements of FDE activity (both in the buffered venoms of the various snakes and in the final FDE preparations), sometimes gave such a wide range of results as to considerably increase the average error of the method. This wide spread was observed, as a rule, between data of the various series of experiments, while the results within the limits of a series tended to be practically identical. The greatest range of data was

observed in viper venom, while in the buffered echis venom the activity values could be divided into two groups. We cannot exclude the possibility that one reason for this variance could be due to the fact that we used dried venom from different batches, which might have varied in methods of collection, in the conditions of the snakes yielding the venom, and in storage time of the material. This circumstance alone, however, could not fully explain the divergent data obtained in our acetone fractionation.

Some factors, apparently, influence the enzymatic activity of protein in each given case—factors difficult for us to determine since the properties of the FDE active protein and the kinetics of the enzyme reactions are both, as yet, practically unstudied problems. Our presumption—that the divergence in data is due to the nature of the protein itself—is further condirmed by the fact that the FME results correlate exactly, although their determination is conducted under the same conditions as the FDE determination. We must, therefore, presume either that the FME is more stable to the various influences of collection and storage, or else that the slightly varying experimental conditions have, but little effect on measurements of its activity.

Our results show some agreement with the data presented by Shnol' et al., [7, 8]; they reported that in their study of the ATP activity of myosin and actomyosin the wide range of data in duplicates of one and the same experiment was conditioned, mainly, by properties of the protein itself.

Our above-mentioned difficulties not withstanding, we can draw certain conclusions, both as to the differing original FDE activity levels of the snake venoms investigated, and as to the suitability of acetone fractionation in purifying the FDE of the various venoms.

Some venoms, apparently, require a certain modification of method to yield satisfactory results; this modification, it seems, should comprise not only changes in the amount of acetone added, but should include some other selected experimental conditions—(temperature of the acetone, concentration of venom, etc.).

Our statement that FDE activity is 200-300 times higher than FME activity calls for some qualification, since the two enzymes were tested on different substrates, and this could have affected their absolute quantities. We note that data on FDE activity–obtained by FDE acting on the calcium salt of di (paranitrophenyl) phosphoric acid—can give but a relative concept of FDE activity when the substrate is DNA. Khorana [9] specifically indicates that the hydrolysis rate of thymidyl oligonucleotides by FDE considerably surpasses the rate of the nitrophenol derivatives' hydrolysis by the same enzyme. It is natural to presume that this difference would be manifest, in one form or another with the action of FDE on a high-molecule DNA.

In evaluating the comparative FDE activity of various venoms, we cannot, with full confidence, state that this same relationship would hold for the action of these venoms on DNA, since due to the possible specificities of FDE from varying sources, the relative rates of hydrolysis, could in this case, be some what different.

SUMMARY

The enzymatic activity was studied venoms derived from snakes occurring mainly in the USSR (Vipera lebetina, Echis carinatus, Naja oxiana) and of the South American rattle snake. All the venoms tested exhibit a phosphodiesterase and 5'-nucleotidase activity.

In the venoms of all the four snake species phosphodiesterase activity is 200-300 times higher than that of 5'-nucleotidase. The highest phosphodiesterase activity was found in Vipera lebetina, the least one in Echis carinatus. The 5'-nucleotidase activity was approximately similar in all venoms. The yield of phosphodiesterase upon acetone fractionation amounts to 8-11% in Vipera, Echis and rattle snake, but does not exceed 2.5% in Naja. Separation of phosphodiesterase from 5'-nucleotidase by acetone fractionation proved satisfactory only in the Echis venom.

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ROLE OF SULFHYDRYL GROUPS IN POLYMERIZATION OF ACTIN

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A number of papers have been published over the past few years, devoted to he study of polymerization of actin, and to the identification of the functional groups taking part in this process. The first indication that Sh-groups were concerned in the transformation of G-actin into F-actin emerged from the work of Kuschinsky and Turba [1], who showed that polymerization of actin is inhibited by Salyrgan. It may be concluded from the findings of Mommaerts [2], Kominz et al. [3], and Barany [4], that 5-6 cysteine residues are present per molecule of actin (monomer, mol. wt. 70,000). Barany [4], and Barany et al. [5], determined the number of SH-groups present in F- and G-actin, in relation to polymerization, and found that the free SH-group content of F-actin was only half that of G-actin. Salyrgan titration showed the presence of four SH-groups in $57 \cdot 10^3$ g of G-actin, and of only two such groups in the same weight of F-actin, at pH 6.5-7.0. They also showed that acetylation or formylation of NH_2-groups led to reversible depolymerization of actin. The authors hence concluded that a primary factor in the transformation of the globular to the fibrous form of actin was hydrogen bonding between a SH-group of one actin molecule and an NH_2-group of another.

Since some doubt may attach to the validity of the conclusions of Barany et al. [4, 5], we undertook the re-examination of the role of SH-groups in the process of polymerization of actin. In contradistinction to these authors, we preferred to use direct titration of SH-groups, using the inorganic reagents mercuric chloride and silver nitrate for the purpose.

METHODS

The analytic method used in the present research was largely the same as that described by us in an earlier paper [6]. Actin was prepared from rabbit muscle by the method of Tsao [7]. Actin was polymerized by adding to its solution KCl and $MgCl_2$, to final concentrations of 0.1 M and 0.001 M, respectively. These salts were added in all experiments involving F-actin. The degree of polymerization of actin was assessed from the change in the viscosity of its solutions, as measured in an Ostwald viscometer, with a water flow-time of 40 sec at 20°.

For the amperometric titration of SH-groups we used a rotating platinum electrode, in the earlier described apparatus [6]. As the reference electrode we took Samuelson and Brown's saturated barium electrode [8], as recommended for this purpose by Benesch et al. [9]. The electrode potential (− 0.1 v) permits the titration of both silver and mercury.

Sulfhydryl groups were titrated with $AgNO_3$ in tris buffer, pH 8. Titration with $HgCl_2$ was effected in borate buffer of various pH values (from pH 7 to pH 10), in the presence of KCl or NH_4OH, in tris buffer, pH 8.7, and in ammonia buffer, pH 10. The $AgNO_3$ solution was titrated against standard 0.1 M NaCl solution. A highly purified specimen of mercuric chloride was taken for direct preparation of standard solutions. We used 15 ml samples for titration.

The validity of applying the above described methods to the elucidation of the given problem merits special consideration. The trustworthiness of the argentometric titration method arouses no particular doubts, for the given purpose. It is known that argentometric titration of, for example, glutathione, in an ammonia buffer may give somewhat low results [9, 10], because of the instability of the glutathione–silver complex, but this source of error, if it exists in the given case, cannot be of significance, since we are concerned with the determination of a number of SH-groups differing by a factor of 2.

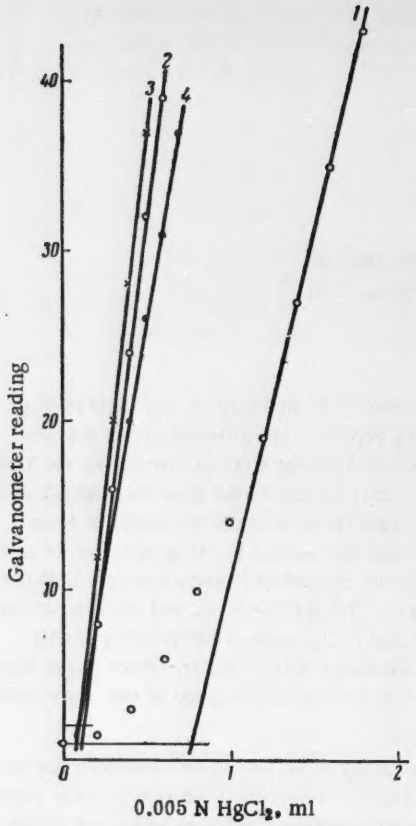

Fig. 1. Amperometric titration of G-actin. For the titration we took 4.85 mg of protein. The solution was titrated: 1) in borate buffer pH 9.3; 2) in borate buffer, pH 9.3, containing 0.5 M KCl; 3) in ammonia buffer, pH 10; 4) in tris buffer, pH 8.7.

Greater caution is needed for the interpretation of the results of mercurimetric titration. Mercuric chloride belongs to the category of readily soluble, but weakly dissociated compounds. Its dissociation proceeds in two steps:

$$HgCl_2 \rightleftarrows HgCl^+ + Cl^- \rightleftarrows Hg^{++} + 2\,Cl^-$$

Although the concentration of free Hg^{++} ions is, in practice, negligibly small, the presence of bivalent mercury nevertheless introduces certain complications into amperometric titration. Hughes [11, 12] has shown that serum albumin (A-SH) reacts with mercury salts to yield two types of mercaptalbumin derivatives—the monomeric form (R-S · HgCl) and the dimeric form (R-S · · Hg · S-R), which can coexist, and which are interconvertible. The dynamics of these transformations have been studied by Edsall [13], who found that the amounts of monomer and dimer formed were a function of the stoichiometric ratio $[HgCl_2]/[A\text{-}SH]$. The dimeric form predominates when this ratio is 0.5, and the monomeric form when it is equal to unity.

The reaction between mercuric chloride and albumin proceeds in three steps: monomer is formed in the first, it is transformed into dimer in the second, while monomer again begins to predominate in the third. In agreement with this formulation, Ingram [14] has shown that in the amperometric titration of SH-group of hemoglobin one atom of mercury is capable of reacting with two SH-groups.

A second complicating factor is the definite affinity of mercury for amino, amido, and imidazolyl groups, as has been shown by Haarmann [15]. It is owing to this property that it is possible, under appropriate conditions, to titrate all of the peptide groups of peptone. We have also found that amperometric titration of acetone or ascorbic acid with mercuric chloride is possible (the reaction mechanism differs from that of titration of proteins; with acetone, for example, $HgCl_2$ reacts with methyl groups, the reactivity of which is enhanced by the carbonyl group).

Notwithstanding these findings, conditions exist under which only one of the valences of mercury reacts with SH-groups. The Hg^{++} ion has an affinity for halide and ammonium ions, and this may, as was shown by Kolthoff et al. [10], be made use of in the titration of protein SH-groups, for the exclusion of side-reactions of $HgCl_2$. This may be achieved in practice by adding KCl, tris, or NH_4OH to the reaction mixture. As regards Cl^-, the effect might be based on suppression of the second dissociation step of mercuric chloride: $HgCl_2 \rightarrow Hg^{++} + 2Cl^-$. However, the similar effect of NH_4OH or tris suggests the existence of some common mechanism applying to all three reagents, and that this would be of a different nature to the above, and would depend on complex formation. In accordance with this view, we can regard all three reagents (KCl, NH_4OH, and tris) as complexing agents, and their effect as being one of complex formation. Presence of complexing agents would fully exclude the formation of dimeric forms of protein mercaptides of the type RS · Hg · SR, as well as the reaction of $HgCl_2$ with functional groups other than SH. The presence of these additives is superfluous for the titration of F-actin, since, as was specified above, the titration solution always contained 0.1 M KCl and 0.001 M $MgCl_2$. On the other hand, however, in the titration of G-actin the presence of KCl is undesirable, since it promotes transformation of G-actin into F-actin, and here the addition of ammonia is necessary; hence ammonia buffer is used. The resulting mercury—ammonia complex reacts with SH-group as a univalent reagent only.

The curves of Fig. 1 illustrate the results of amperometric titration of actin in media which exclude the possibility of side-reactions (viz., with amino, amido, imidazolyl, and other groups), and in media in which such reactions are not excluded. The solutions were titrated at alkaline pH (pH 8.7-10), at which Hg^{++} very readily enters

into reaction with amino, amido, and other such groups of protein molecules. In the absence of Cl^- 4.85 mg of actin, dissolved in buffer solution of pH 9.3, binds 0.76 ml of 0.005 M $HgCl_2$, which corresponds to 56 equivalents of mercury per molecule of actin (of mol. wt. 70,000). This exceeds the number of SH-groups per molecule by a factor of ten (see below). When KCl is added to the borate buffer to a final concentration of 0.5 M, the amount of $HgCl_2$ reacting at once falls to a level corresponding with the actual SH-group content of actin (curve 2). The same was found for titration of actin in ammonia buffer, pH 10, or in tris buffer, pH 8.7. The curves of Fig. 1 clearly demonstrate the importance of complex-formation in the titration of SH-groups of actin, and they also show that the results obtained when KCl, NH_4OH, or tris are used as complexing agents are in agreement with each other.

It may hence be taken as established that the quantitative analytical methods used by us for the determination of the SH-group content of actin are sufficiently accurate for the purposes of the given research.

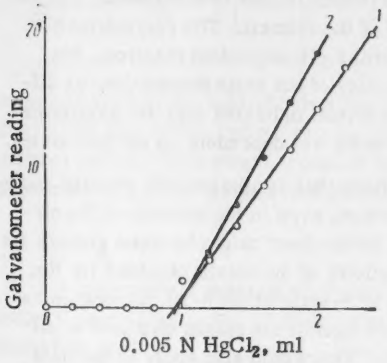

Fig. 2. Amperometric titration of SH-groups of actin with mercuric chloride. The solutions titrated contained 59 mg of protein. 1) G-actin: composition of titrated solution—borate buffer, pH 7.0, 0.25 M NH_4NO_3 + 0.08 M NH_4OH (complexing agent) + 59 mg of G-actin; total volume 15 ml, final pH 7.2; 2) F-actin: composition of solution—borate buffer, pH 7.0, 0.6 M KCl + 0.001 M $MgCl_2$ (activating mixture) + 59 mg of F-actin; total volume 15 ml.

EXPERIMENTAL RESULTS

Behavior of SH-Groups in the Transformation of F-Actin to G-Actin

In the experiments of this series the transformation of F-actin into G-actin was stimulated by addition of an activating mixture ((KCl + $MgCl_2$, to final concentrations of 0.1 M and 0.001 M, respectively), and the extent of transformation was derived from measurements of relative viscosity. Barany et al. [4, 5], have shown that the optimum pH for this transformation is from 6.5 to 7.0. We therefore, conducted both the transformation of G-actin to F-actin, and the titration of SH-groups, at pH 7.0-7.2. Curve 1 (Fig. 2) represents the titration of 59 mg of G-actin with mercuric chloride, and curve 2 that of 59 mg of F-actin. It is clearly evident that both forms of actin have the same SH-group content, and that the polymerization process has no effect on the SH-group content of actin, as determined by titration with $HgCl_2$. The absolute SH-group content of one mole of actin monomer (mol. wt. 70,000) amounts to 5.9 moles, which is practically identical with the value found by Barany [4, 5].

The same result was given by amperometric titration of SH-groups of both forms of actin in tris buffer, pH 8.0, with silver nitrate solution (Fig. 3); transformation of G- into F-actin did not cause any change in SH-group content of the protein. The absolute SH-group content of the protein. The absolute SH-group content indicated by argentometric titration was somewhat higher than that found above, viz., 6.8 moles of SH-groups per mole of actin monomer (mol. wt. 70,000). It appears that argentometric titration in tris buffer gives somewhat high results, due to the instability of the complex formed between tris and $AgNO_3$.

Thus the transformation of the G- to the F-form of actin did not appear to involve any change in the SH-group content of the protein, as indicated by the results of titration with mercuric chloride or silver nitrate.

Changes in the Viscosity of F-and G-Actin Following Reaction of Their SH-Groups With Mercury

These experiments are closely bound up with the preceding ones. If we are unable to find any change in the SH-group content of actin in association with its transformation from the G- to the F-form, then we should not expect to find any depolymerization of F-actin -when treated with mercaptide-forming reagents, with the reservation that the reagent should be sufficiently specific in its action. The considerations advanced above under "Methods" allow of the assumption that $HgCl_2$ satisfies this requirement, under the appropriate conditions (and these conditions were observed in our experiments). Fig. 4 illustrates the changes taking place in the viscosity of F-actin when all of its SH-groups are combined with mercury, under conditions ensuring the specificity of the reaction. The relative

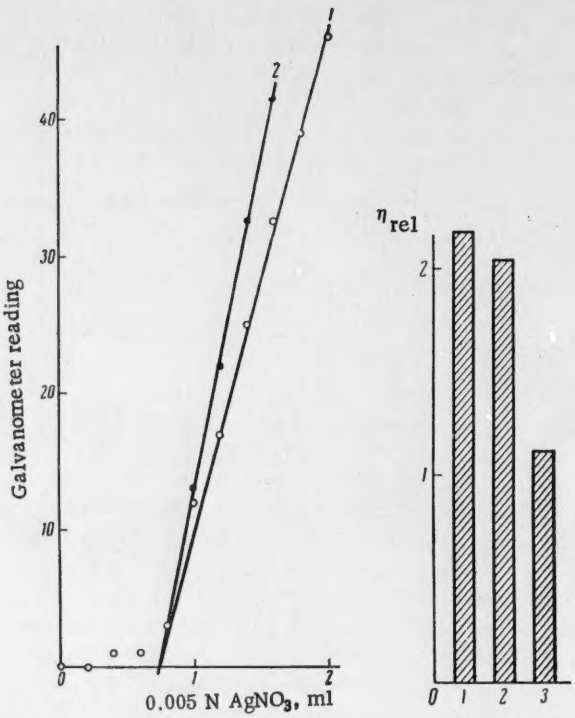

Fig. 3. Amperometric titration of SH-group of actin with silver nitrate. The solutions titrated contained 45.1 ml of protein. 1) G-actin: composition of titrated solution—tris buffer, pH 8, 45.1 mg of G-actin; total volume 15 ml; 2) F-actin: composition of solution—tris buffer, pH 8, 0.01 M KCl + 0.001 M $MgCl_2$ (activating mixture), 45.1 mg of F-actin; total volume 15 ml.

Fig. 4. Viscosity of F-actin solutions, before and after reacting its SH-groups with mercury. 1) Viscosity of F-actin before reaction of its SH-groups with mercury: composition of solutions—borate buffer, pH 7, 0.1 M KCl, 0.001 M $MgCl_2$, actin; 2) viscosity of F-actin after reaction of its SH-groups with mercury: composition of solutions—borate buffer, pH 7, 0.1 M KCl, 0.001 M $MgCl_2$, actin; 3) viscosity of G-actin: composition of solutions—borate buffer, pH 7, actin.

viscosity of F-actin amounted to 2.18 before treatment with mercuric chloride, and it fell only to 2.04 following treatment, whereas the viscosity of G-actin amounted to 1.12. In agreement with expectation, therefore, although $HgCl_2$ gives rise to mercaptide formation, this does not affect the viscosity of F-actin. It should be remembered that Salyrgan causes depolymerization of F-actin, as was shown by Kuschinsky and Turba [1], and this underlines the radical differences in the behavior of the two mercaptide-forming reagents—and Salyrgan. Barany mentioned that the depolymerizing action of Salyrgan and F-actin was independent of the concentration of the former (which is hard to understand), but depended on the pH of the systems. The polymerization of actin is in general a pH-dependent reaction. We measured the viscosity of our actin preparations at different pH, and our results indicated that the transformation G-actin → F-actin was dependent on pH (see table).

It hence follows that at alkaline pH F-actin undergoes depolymerization, even in the absence of depolymerizing agents. While there might be some grounds for questioning the validity of the results obtained for the SH-group content of F-actin at pH 8-10, no such objection could be raised against the results obtained at pH close to neutrality. This would also apply to the data illustrated in Fig. 2.

DISCUSSION OF RESULTS

It follows from the above data that the transformation of G-actin to F-actin is not associated with any change in the content of SH-groups, titratable with $HgCl_2$ or $AgNO_3$. This conclusion seems to be sufficiently established by our findings, and is further supported by the inability of $HgCl_2$ to depolymerize F-actin, as was shown by viscosity measurements. Incidentally, as regards $HgCl_2$, perhaps we should have taken into consideration a possibility which, at first sight, might appear to render our experimental results less convincing. It is conceivable that, following formation of a mercaptide of the type RS · HgCl ⇌ RS · Hg^+ + Cl^-, the second valency of mercury would be free to react with amino groups of the polypeptide chain, or with substituted amido groups of the peptide linkages of a second molecule of actin. In the latter case, a structure of the type

R-S · Hg-N-C-R would be obtained. The possibility of formation of a hydrogen bond of the type R-SH. . . NH_2-R
  | ||
  R O

has been admitted by a number of authors [16], and, as has been mentioned, Barany [4, 5] attributes a major role in the polymerization of actin to such bonds. If this view is correct, then it might be assumed that the formation of a covalent bond between SH-groups and the nitrogen of amino or amido groups, through Hg, as has been mentioned before, would take place between the same sities as those of the pre-existing hydrogen bonds of the type R-SH. . . . . .NH-R (the distance between H and N may be supposed to be small in such cases), and that the mercury bridges replaces the hydrogen one, in a functional sense. If this were to be the case, it is quite possible that the viscosity of F-actin would remain unchanged. The whole assumption should, nevertheless, be rejected, since our experimental

Effect of pH on the Transformation of G-Actin into F-Actin. [Composition of medium: Borate buffer + activating mixture (0.1 M KCl + 0.001 M $MgCl_2$)

pH	Rise in viscosity after adding activating mixture to G-actin	
	relative viscosity	%
7,1	2,02	100
7,6	1,79	89
8,1	1,18	58
8,6	0,74	36
9,0	0,15	7
10,0	0	0

conditions were such as to exclude the possibility of reaction of Hg^{++} with amino or amido groups. Moreover, our results would still differ from those of the experiments with Salyrgan, since the SH-groups of F-actin are also fully titratable in this variant.

As for the data obtained by other authors, working with Salyrgan, we can only suppose that much depends on the greater size of the organic part of the Salyrgan molecule, and on the presence in it of other reactive groups. From this viewpoint, the inaccessibility to Salyrgan of two of the SH-groups of the F-actin molecule may be related to steric hindrances resulting from changes in the tertiary structure of this protein. Sites which cannot be approached by the relatively large Salyrgan molecule may well be readily accessible to mercury ions. Similarly, the effect of Salyrgan on the viscosity of F-actin may be related to the action of functional groups of its molecule, rather than to mercury. In general, we share the somewhat sceptical view of the role of SH-groups in enzyme activity and in protein structure, held by Boyer [17], and we think that in the past undue importance has not infrequently been attributed to this role, on the basis of evidence of an indirect nature. This would appear to apply, in particular, to the assumption that SH-groups play an exclusively important part in the polymerization of actin.

SUMMARY

Amperometric titration of G- and F-actin with $HgCl_2$ or $AgNO_3$ shows the presence of about 6 SH-groups per mole of protein (mol. wt. 70,000). The number of amperometrically titratable SH-groups was unchanged by transformation of G-actin to F-actin. The viscosity of F-actin fell by only 12-15% after all of its SH-groups had reacted with $HgCl_2$ at pH 7.0, showing that its degree of depolymerization had scarcely been affected by this treatment. As shown by viscosity measurements, transformation of G- to F-actin depends on the pH of the medium, and is suppressed at alkaline pH.

There is no reliable evidence of the direct participation of SH-groups in the polymerization of actin.

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PATHWAYS OF ENZYMIC FORMATION OF HYDROGEN SULFIDE FROM L-CYSTEINE IN THE LIVER

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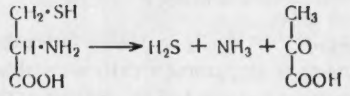
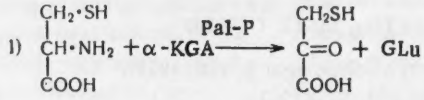
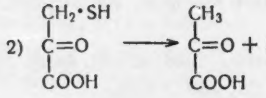
Notwithstanding the number of papers published on the desulfuration of Cys* in animal tissues and in microorganisms, conflicting views are still held regarding the mechanism of this process. The process of enzymic formation of H_2S from L-Cys was first described by Fromageot [1]. The enzyme catalyzing this reaction was named cysteine desulfhydrase. Although different views as to the nature of the products of desulfuration of Cys have been advanced by different authors, most of them believe that these products are H_2S, NH_3, and PA, formed according to the reaction:

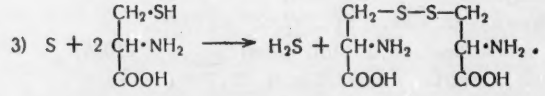


Braunshtein and Azarkh [2] have shown that Pal-P takes part in the process of formation of H_2S from Cys.

A second pathway of desulfuration of Cys has been recognized in recent years [3], involving its transamination with α-KGA or PA, with formation of β-mercaptopyruvic acid, which then breaks down to give PA and elemental sulfur, the latter then being reduced by excess of cysteine giving H_2S:







This pathway of formation of H_2S also involves the participation of Pal-P.

Chatagner et al. [4], working in Fromageot's laboratory on the effect on L-Cys of liver extracts from rats which had been fasted for 24 hr, or of liver extracts which had been dialyzed for 24 hr, found that anaerobic formation of H_2S from Cys was not accompanied by NH_3 formation, and that addition of α-KGA stimulated the process. The formation of NH_3 reported by some authors was attributed to the action of glutamate dehydrogenase on Glu arising during the transamination reaction [4, 5]. Transamination between Glu (or Cys) and PA formed from mercaptopyruvate leads to production of alanine, which was identified in Fromageot's laboratory as being one of the products of reaction of Cys.

* The following abbreviations are used in this paper: Cys—cysteine, PA—pyruvic acid, α-KGA—α-ketoglutaric acid, Glu—glutamic acid, Pal-P—pyridoxal phosphate, DEAE-cellulose—diethylaminoethylcellulose, EDTA—ethylenediaminetetra-acetic acid.

TABLE 1. Degradation of Cysteine in the Presence of Dialyzed and Undialyzed Rat-Liver Extracts (as μ moles per gram of liver)

No. of expt.	Enzyme preparation	Cys	α-KGA	Pal-P	ΔH_2S	ΔNH_3
1	Liver extract	+	−	−	20,5	13,4
		+	+	−	20,5	12,9
2	» »	+	−	−	24,5	14,5
		+	−	+	28,7	20,5
3	Extract of liver of rats receiving vitamin B_6	+	−	+	34,0	21,3
		+	+	+	30,0	20,1
4	Liver extract dialyzed for 15 hr	+	−	−	3,2	2,7
		+	+	−	7,6	3,2
5	Liver extract dialyzed for 17 hr	+	−	−	1,05	0,75
		+	−	+	5,7	6,0
		+	+	−	5,0	0,7
		+	+	+	9,2	2,1

It was thought to be of interest to ascertain whether animal tissues contain an enzyme catalyzing direct desulfuration of Cys, with production of NH_3, and to evaluate the significance of indirect desulfuration catalyzed by cysteine transaminase and mercaptopyruvate desulfurase. The present research is devoted to the study of these problems.

EXPERIMENTAL

Formation of NH_3 and H_2S from Cys in the presence of liver extracts. The experimental material was taken from rats, which had been maintained on an ordinary diet, supplemented in some cases by daily injections of 100 μg of pyridoxine. The livers were homogenized, after cooling, for 2 min in four volumes of cooled distilled water, and the homogenate was centrifuged at low temperature (2500 r.p.m. for 20 min). To the experimental systems, of final volume 5 ml, containing 1-2 ml of liver extract and phosphate buffer (0.1 M, pH 7.3), we added 50 μ moles of L-Cys, 25 μ moles of α-KGA, and 60 μg of Pal-P. The systems were incubated anaerobically in specially designed flasks [2] for 2-3 hr at 37°, in an atmosphere of purified nitrogen. Ammonia was determined in the protein-free filtrates by the method of Speck [6], and H_2S was determined by Fogo's method [7]. The amount of substrate which had reacted was expressed as μ moles of H_2S and NH_3 formed per gram of fresh liver tissue. The results are presented in Table 1.

We found that both NH_3 (13-21 μ moles) and H_2S (20-28 μ moles) were formed during incubation of L-Cys with liver extracts; the increment in H_2S content (ΔH_2S) always exceeding that in NH_3 content (ΔNH_3). Production of NH_3 and of H_2S from L-Cys was unaffected by addition of α-KGA (Table 1, experiment 1 and 3); addition of Pal-P to systems containing Cys or Cys + α-KGA caused a pronounced rise in NH_3 and H_2S production (Expt. 2). Dialysis of the liver extracts against 0.01% $NaHCO_3$ for 15-24 hr led to a considerable fall in production of H_2S and NH_3 from Cys (Expts. 4 and 5). When α-KGA was added to such systems H_2S production was raised, but NH_3 production was unaffected (Expt. 4). Addition of 60 μg of Pal-P to systems containing dialyzed extract greatly increased the yields of H_2S and NH_3, which were produced in equimolecular amounts (Expt. 4). It was of interest to compare the effect of α-KGA on production of H_2S from Cys in the presence of dialyzed or undialyzed liver extracts with the intensity of the transamination reaction between Cys and α-KGA.

The transamidation reaction was followed from the results of determinations of Glu, by means of paper chromatography. Portions of filtrate were applied to the paper, and the spot was treated with 30% H_2O_2. Sulfur-containing amino acids were thus oxidized to products giving low R_f values. The solvent system used for development was butanol–CH_3COOH–water (40 : 10 : 50), and the chromatograms were sprayed with 0.2% ninhydrin.

It appears from the chromatograms (Fig. 1, A) that Glu and Ala appeared in the systems when α-KGA alone was added to the liver extract. The amount of Glu produced was only slightly affected by the further addition of Pal-P and Cys. With dialyzed liver extract, Glu was formed only when both Cys and α-KGA were both present, and the yield was higher when Pal-P was also added (Fig. 1, B).

TABLE 2. Purification of Rat-Liver "Cysteine Desulfhydrase." [Units of activity (U) per μ mole of NH_3]

Stage of purification	Enzyme preparation	Vol. ml	Total protein content, mg	Total activity, U	Specific activity, U/mg protein	Yield, %
1	Initial liver extract	185	8325,0	499,5	0,06	100
2	Extract of acetone powder	160	371,2	419,0	1,13	84
3	0.05 M KCl eluate from DEAE-cellulose	125	40,0	200,0	5,00	40

TABLE 3. "Cysteine Desulfhydrase" and Cystathionase Activities with Three Substrates (ΔKeto acids in μ moles per mg of protein)

No. of expt.	Enzyme preparation	Cysteine	Cystathionine	Homoserine	Ratios of activities
1	Extract of acetone liver preparation	0,70	3,41	4,20	17 : 81 : 100
2	The same	0,92	2,96	4,33	21 : 68 : 100
3	"Cysteine desulfhydrase"	1,36	8,75	9,27	15 : 94 : 100
4	" "	0,80	7,00	9,00	8 : 77 : 100
5	Cystathionase	1,60	7,60	13,40	12 : 57 : 100
6	"	1,13	8,38	12,25	9 : 68 : 100

From these two series of experiments we conclude that production of H_2S (without rise in NH_3 content), augmented by addition of α-KGA, is the result of indirect desulfuration of Cys, with intermediary formation of β-mercaptopyruvate, as described by Meister and by Chatagner et al. [3, 4], whereas production in equimolar amounts of H_2S and NH_3, which is not stimulated by addition of α-KGA, is due to direct decomposition of Cys by a desulfhydrase type of enzyme.

Isolation and purification of "cysteine desulfhydrase." We developed the following procedure for the purification of the enzyme catalyzing the reaction of production of H_2S and NH_3 from Cys; the name "cysteine desulfhydrase" has provisionally been given to this enzyme.

Rat-liver extract was shaken energetically with an equal volume of chloroform (previously cooled to −10°) for 30 min, and the emulsion was centrifuged at 0°. Much of the ballast protein separated out during this manipulation, while the enzyme remained in the aqueous layer [8]. To the latter we added two volumes of acetone cooled to −10°, and the resulting precipitate was collected on the centrifuge, washed with cooled acetone, and air-dried. The resulting powder retained its activity for many months, when stored at low temperature in a desiccator.

The acetone powder was extracted with two volumes of water, and the extract was treated with DEAE-cellulose. Column fractionation with this anionite proved to be impracticable, because of the instability of "cysteine desulfhydrase" under such conditions. We therefore suspended DEAE-cellulose in the extract, containing borate buffer (0.005 M, pH 8.0), and the suspension was stirred for 5 min, when the enzyme was totally adsorbed on the anionite. The anionite was then collected on a Buchner funnel, and washed with small portions of the same borate buffer. The enzyme was extracted by suspending the washed anionite successively in borate buffer solutions (0.005 M, pH 8.0) containing increasing concentrations of KCl (0.05, 0.1, and 0.2 M), collecting the eluate on the filter pump after each operation. We found that cysteine desulfhydrase activity was eluted by borate buffer containing 0.05 M KCl, and that β-mercaptopyruvate desulfhydrase activity was eluted at the higher KCl concentrations.

The data of Table 2 show that the specific activity of the preparations rose by a factor of 80-90 as a result of the purification procedure, with an over-all yield of activity of 40%.

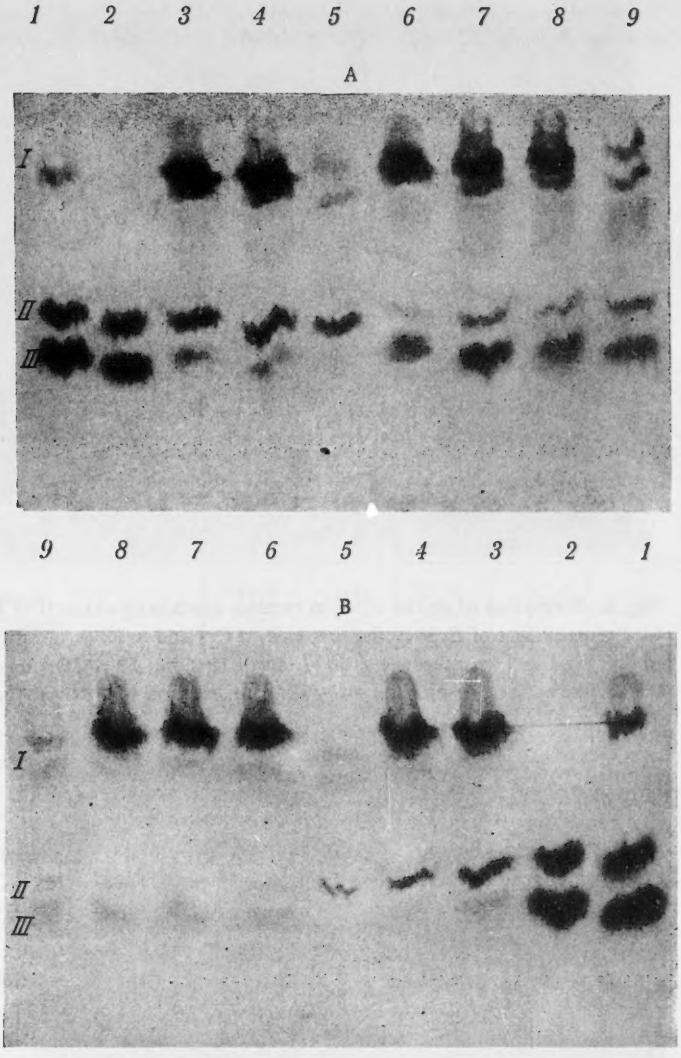


Fig. 1. Formation of amino acids in systems containing (A) undialyzed, (B) dialyzed, liver extracts with Cys and α-KGA. 1 and 2—Reference spots (I—Cys; II—Glu; III—Ala); 9—tissue; 8—tissue + Cys; 7—tissue + + Cys + Pal-P; 6—the same, + EDTA; 5—tissue + α-KGA; 4—tissue + α-KGA + Cys; 3—tissue + α-KGA + Cys + Pal-P.

Properties of purified "cysteine desulfhydrase." We assessed the cysteine—glutamate transaminase activity of our purified "cysteine desulfhydrase" preparation, by the paper chromatography method. It is evident from Figure 2 that only insignificant production of Glu took place in systems containing acetone powder extract with Cys + KGA, and that practically no Glu was formed in systems containing cysteine desulfhydrase of the highest specific activity (Fig. 3). Nor could we detect any glutamate dehydrogenase activity, responsible for production of NH_3 from Glu, and mercaptopyruvate desulfhydrase activity was similarly vanishingly small.

Marsuo and Greenberg [9] obtained a crystalline preparation of cystathionase from rat liver, which also actively decomposed homoserine or Cys, with production of NH_3 and the appropriate α-keto acids. When examining the substrate specificity of our "cysteine desulfhydrase" preparations we found that they attacked not only Cys, but also homoserine and cystathionine, giving NH_3 and α-keto acid from both of these substrates. We made a quantitative comparison of the activities of purified "cysteine desulfhydrase" and of cystathionase prepared according to Marsuo and Greenberg, with the three substrates Cys, homoserine, and cystathionine (Table 3).

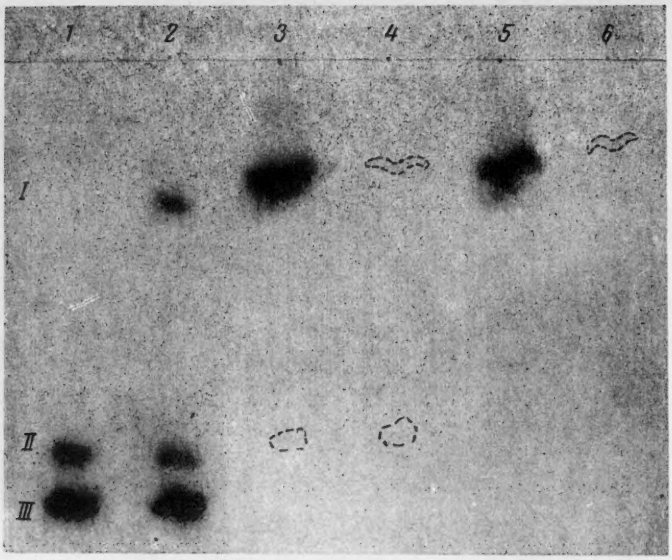


Fig. 2. Formation of amino acids in systems containing extracts of acetone preparations of liver in the presence of Cys and α-KGA. In this, and in Fig. 3: 1 and 2—reference spots (I—Cys; II—Glu; III—Ala); 6—enzyme; 5—enzyme + Cys; 4—enzyme + α-KGA; 3—enzyme + α-KGA + Cys.

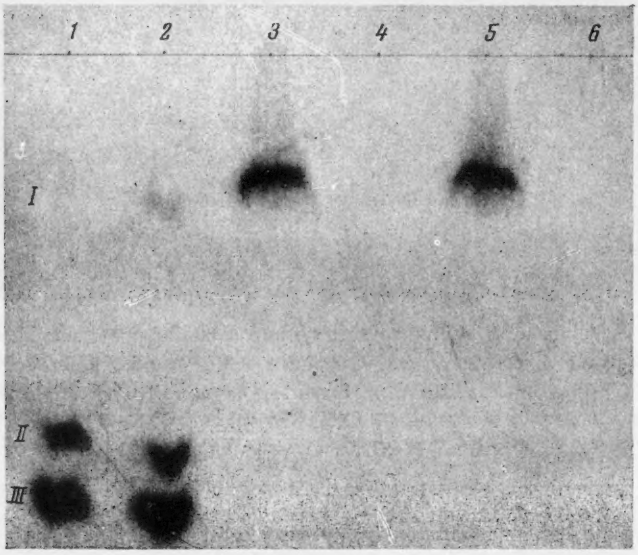


Fig. 3. Formation of amino acids in systems containing 0.05 M KCl eluate of DEAE-cellulose in the presence of Cys and α-KGA.

Our results (Table 3) show that both enzymes display the greatest activity with homoserine as substrate, and the least with Cys; "cysteine desulfhydrase" is, however, somewhat more active with respect to cystathionine.

The "direct" desulfuration of Cys by liver extracts or enzyme preparations derived therefrom may thus be largely, if not exclusively, attributable to the multifunctional enzyme cystathionase. Further investigations would be required to establish whether a narrowly specific cysteine desulfhydrase is present in liver, or in liver preparations, distinct from cystathionase.

TABLE 4. Effect of EDTA on the Enzymic Degradation of Cys, Cystathionine, and Homoserine (ΔKeto acids, in μmoles per mg of protein; concentration of substrates $1.6 \cdot 10^{-10}$ M)

No. of expt.	Enzyme preparation	Cysteine	Cystathionine	Homoserine
1	"Cysteine desulfhydrase"	1,36	8,75	9,27
2	The same, + EDTA, $2.2 \cdot 10^{-3}$ M	1,02	9,70	13,25
3	Cystathionase	1,36	5,36	6,26
4	The same + EDTA, $2.2 \cdot 10^{-3}$ M	1,90	5,60	6,60

TABLE 5. Effect of Cycloserine on the Activity of "Cysteine Desulfhydrase" and of Cystathionase (Keto acids, in μmoles per system; concentration of substrates $1.6 \cdot 10^{-2}$ M)

Substrate	Cystathionase			"Cysteine desulfhydrase"		
	activity without inhibitor	with cycloserine, 10^{-4} M	inhibition, %	without inhibition	with cycloserine, 10^{-4} M	inhibition, %
Homoserine	16,7	1,4	91,8	5,5	1,1	80,7
Cystathionine	9,5	4,3	54,8	7,2	4,0	44,0
Cysteine	2,0	1,4	30,0	1,2	1,1	3,5

Important, though not conclusive, evidence as to the identity or nonidentity of different enzyme systems may be derived from the examination of the action thereon of "effectors," i.e., of substances which activate or inhibit enzyme systems. According to the literature, EDTA activates cystathionase [9], but inhibits cysteine desulfhydrase [10]. Bearing this in mind, we examined the action of $2.2 \cdot 10^{-3}$ M EDTA on the activity of our preparations of "cysteine desulfhydrase" and cystathionase. The enzyme solutions were first incubated with EDTA (and, in subsequent experiments, with other inhibitors), and we then added the substrates. The data of Table 4 show that with "cysteine desulfhydrase" EDTA slightly inhibits decomposition of Cys, but augments that of homoserine and cystathionine. With cystathionase, EDTA activated the reaction with all three substrates, most of all with Cys.

The antibiotic cycloserine is a powerful inhibitor of transaminases and of certain other enzymes requiring pyridoxal as a coenzyme [11, 12]. We examined the effect of cycloserine on the activity of the multifunctional enzymes prepared by us. We added DL-cycloserine to systems containing "cysteine desulfhydrase" or cystathionase, with Cys, homoserine, or cystathionine as substrates. The enzyme preparations were first incubated with 10^{-3}-10^{-5} M DL-cycloserine, before adding the substrates ($1.6 \cdot 10^{-2}$ M). The α-keto acid content of the systems was determined after anaerobic incubation, by the method of Friedemann and Haugen [13]. It appears from the data of Table 5 that the inhibitory effect of DL-cycloserine (10^{-4} M) was greatest, with both enzyme preparations, with homoserine, and least with Cys. Cycloserine (10^{-4} M) caused 30% inhibition in cystathionase—cysteine systems, as compared with only 3.5% inhibition of "cysteine desulfhydrase."

It has been reported [4, 14] that arsenate interferes with production of H_2S from cysteine in animal tissues. It was of interest to ascertain to what extent this effect is related to inhibition of "cysteine desulfhydrase" or cysteine—glutamate transaminase activities.

Rat-liver extracts were preincubated with Na arsenate (25 μmoles) for 10 min, after which we added Pal-P (10 μg per system) and substrate (50 μmoles of Cys, or 50 μmoles of Cys + 25 μmoles of α-KGA). The following results were found for the ΔH_2S and ΔNH_3 of protein-free filtrates:

Composition of systems	ΔH_2S		ΔNH_3	
	without inhibitor	with arsenate	without inhibitor	with arsenate
Liver extract + 50 μmoles Cys	34.0	1.3	21.3	18.5
Liver extract + 50 μmoles Cys + 25 μmoles α-KGA	30.0	0	20.1	19.8

It is hence evident that arsenate inhibits H_2S formation in liver extract–cysteine systems, but has practically no effect on NH_3 formation, whether in the presence or the absence of α-KGA. These findings are in accordance with those reported by other authors [4, 14]. Arsenate does not inhibit either the transamination reaction with cysteine, or its direct desulfuration; the H_2S evolved reacts with arsenate to give stable, insoluble arsenic sulfide, the effect of which is to interfere with the analytical determination of H_2S.

DISCUSSION OF THE RESULTS

For the elucidation of the mechanism of anaerobic desulfuration of Cys in the liver it was necessary, in the first place, to ascertain whether H_2S can, as some workers hold [4, 5], be formed only by an indirect pathway, of which the first step is transamination between Cys and α-KGA, or whether direct desulfuration of Cys, simultaneously with elimination of NH_3, can also take place.

It is evident from our findings that anaerobic decomposition of Cys in the presence of liver extracts is associated with production of NH_3, the magnitude of ΔNH_3 always being considerably less than that of ΔH_2S. We hence concluded that two parallel processes of H_2S formation coexisted in rat liver, one of these processes not involving NH_3 production. This conclusion was fully supported by the results of our experiments on the decomposition of Cys by dialyzed liver extracts, and on the behavior of purified desulfurating enzyme. We found that production of NH_3 and H_2S from Cys was greatly reduced when the liver extracts were first dialyzed for 15-17 hr against dilute $NaHCO_3$ solution. Enzymic activity was restored by addition of Pal-P to the dialyzed enzyme, and NH_3 and H_2S were then formed in equivalent yields. Inactivation of the enzyme became irreversible with more prolonged dialysis (24 hr). The enzyme system of which the first reaction is that of transamination between Cys and α-KGA was more resistant to prolonged (24 hr) dialysis.

We were able, using the procedures described in the experimental section of this paper, to prepare purified "cysteine desulfhydrase," not containing any cysteine–glutamate transaminase or glutamate dehydrogenase activity.

It may be concluded from our experimental results that H_2S is formed from Cys in the liver by two independent pathways: by direct desulfuration of Cys, which is associated with NH_3 production, and is not stimulated by α-KGA, and by a process of indirect desulfuration, involving transamidation, and not leading to NH_3 production. The relative importance of each of these two pathways of decomposition of Cys would be determined by biological factors, and by the substrate concentrations.

It was next necessary to ascertain whether direct desulfuration of Cys involves the intervention of one enzyme system only. An examination of our purified "cysteine desulfhydrase" preparation showed that its substrate specificity and certain other properties were very close to those of the multifunctional enzyme cystathionase–homoserine deaminase, isolatedby Marsuo and Greenberg [9]. We found, however, that the activities of these two enzyme preparations, isolated from the same tissue, with three different substrates were affected differently by the addition of certain effectors (EDTA, cycloserine). Our findings would be consistent with the view that our "cysteine desulfhydrase" preparations are not fully identical with cystathionase, but also contain some admixture of a more narrowly specific enzyme–"true cysteine desulfhydrase." The possibility of the independent existence of such an enzyme cannot be definitely excluded on the basis of our experimental findings or of published material [10]. It is very probable that direct desulfuration of Cys in animal tissues involves the participation of a number of different desulfurases, differing in their specificities (see also Chapeville's [15] report of one such enzyme, present in the yolk sac of chick embryos).

SUMMARY

Our experiments with liver extracts revealed the existence of two different pathways of formation of H_2S from L-Cys: one involves direct desulfuration of Cys, with production of H_2S and NH_3, while the other, associated with transamidation of Cys, leads to the production of H_2S and Glu. Dialysis of liver extracts leads, in the first place, to inactivation of the direct desulfuration reaction, while the activity of Cys–Glu transaminase, and of subsequent stages of desulfuration, are less affected by dialysis.

A purified preparation of "cysteine desulfhydrase," of specific activity 80-90 times greater than in the initial extract, was isolated from rat liver. This preparation showed no Cys–α-KGA transaminase or glutamate dehydrogenase activity. The substrate specificity of our "cysteine desulfhydrase" preparation closely resembled that of the multifunctional enzyme cystathionase [9]. Both of these enzymes were active with Cys, cystathionine, and

homoserine. Both enzyme preparations are inhibited by the antibiotic cycloserine (10^{-4} M), to the greatest extent with homoserine as substrate, and to the least with Cys.

The activities of the two enzymes towards different substrates are affected differently by the presence of $2.2 \cdot 10^{-3}$ M EDTA. This finding suggests that a number of desulfurating enzymes, differing in their substrate specificities, may participate in the direct enzymic decomposition of Cys.

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All abbreviations of periodicals in the above bibliography are letter-by-letter transliterations of the abbreviations as given in the original Russian journal. *Some or all of this periodical literature may well be available in English translation.* A complete list of the cover-to-cover English translations appears at the back of this issue.

ON CONDITIONS FOR ISOLATION OF PLANT MITOCHONDRIA

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In the study of the significance of structure in cellular function, structural and chemical identification of cell organelles assumes particular importance. Conditions of isolation of a given organelle must be strictly controlled in order to prevent artifacts from arising and also to prevent contamination with other cellular elements.

In this connection, examination under the electron microscope of various cellular fractions obtained by differential centrifugation is of great interest. Such a procedure would, on the one hand, afford more accurate determination of the centrifugal force required for precipitation of a given discrete fraction, and, on the other, provide an opportunity to assess the purity of a preparation. The latter is necessary both in enzymological investigations and in study of the chemical composition of cytoplasmic structures.

In this study, we were especially interested in obtaining mitochondrial preparations free of plastid admixtures. This was necessitated by the fact that the majority of studies of plant mitochondria have been made not with pure fractions, but only with mitochondria-enriched fractions [1].

Goddard and Bonner [2] state that mitochondrial fractions may be obtained from plant homogenates by centrifugation at 1000-2000 g to remove large plastids followed by centrifugation at 6000-10,000 g. Mercer [3] states that they may be obtained by centrifugation at 10,000-15,000 g after preliminary removal of nuclei at 1000-2000 g. In the review by Brown [4], it is indicated that for isolation of mitochondria from leaves it is necessary first to centrifuge at 6000 g, and then at 20,000 g. The conditions which have been employed by various workers to isolate plant mitochondria are given in Table 1.

As the table shows, the first centrifugation has been carried out at 500 g for 5 min, and also at 18,000 g for 0.5 min. The following centrifugation to separate the mitochondrial fraction has also been performed under various conditions—from 7000 g for 10 min to 25,000 g for 30 min. Thus, some workers have used greater forces for the initial fractionation than were used by others for isolation of the main body of mitochondria. Unfortunately, final preparations were not examined microscopically in every case and dimensions of the particles obtained are not, therefore, always available, but from the existing data it is seen that particles obtained by the various workers differ considerably in size.

The question arises as to whether these mitochondrial fractions are not contaminated with plastids, especially when the initial fractionation was carried out at 500 g, and also as to the reason for such widely different regimes for carrying out the final isolation (from 7000 g for 10 min to 25,000 g for 30 min). Published information and personal observations indicate that as yet the conditions necessary for isolation of plant mitochondria have not been clearly established.

With the purpose of obtaining mitochondria free of plastid contaminants, by differential centrifugation, preparations were checked by examination in the electron microscope. Parallel checks of a biochemical nature were made by determination of succinoxidase activity. Such activity is an indication of intact mitochondrial structure.

METHODS AND RESULTS

Peas (Pisum sativum), variety Lincoln, were grown in tap water to 7-10 days of age. Green and etiolated plants were severed 1 cm above the paraffin disk. Leaves and stems were ground in a mortar in the presence of a medium containing sucrose (0.5 M), EDTA (0.005 M), and phosphate buffer (0.006 M, pH 7.3-7.4). All operations were carried out in a cold room at ~ 4°. The brei was pressed through cheesecloth and the homogenate then made up with the grinding medium to a volume corresponding to 1 ml per g original wet weight (usually 100 ml homogenate obtained from 100 g plant tissue). The homogenate was then fractionated by differential centrifugation to

TABLE 1. Centrifugation Regimes Employed by Various Workers to Isolate Mitochondria from Plant Homogenates

Plant material	First centrifugation, prior to isolation of mitochondria		Isolation of mitochondrial fraction		Medium, M	Particle diameter, μ	Authors
	centrifugal force	min	centrifugal force	min			
Tobacco leaves	1000	7	10 000	30	0,4		Pierpoint [15]
" "	120	10	19 000	20	0,15—0,5	0,3—1	Du Buy [6]
" "	2200	20	15 000	30	0,5	≥ 1	McClendon [7]
Pea seedlings	2900	10	7000	10	0,42		Forti et al [8]
" "	1700	15	7500	30	0,4	1	Davies [9]
Arachis cotyledons	18 000	0,5	18 000	20	0,5	4—0,2	Kmetic and Newcomb [10]
Lupine seedlings	1000	15	25 000	30	0,15—0,6	—	Honda et al. [11]
Broad bean leaves	1 000	12	16 000	15	0,3	0,5	James and Das [12]
Castor bean seedlings	500	5	18 000	30	0,5	1—2	Beevers and Walker [13]
Mung bean seedlings	500	5	10 000	15	0,4	0,5—2	Millerd [14]

yield 6 fractions: a—2300 g, 5 min; b—3800 g, 5 min; c—7800 g, 5 min; d— 7800 g, 15 min; e—11,000 g, 15 min; f—16,000 g, 15 min.

Precipitates were thoroughly washed with the grinding medium one time under the same conditions employed in the original fractionation. Washed fractions were suspended in 0.5 M sucrose. Volume of the suspending sucrose medium was usually adjusted to $^1/_{10}$ that of the original homogenate. Determination of succinoxidase activity was made by a method described previously [15].

Suspensions to be examined in the electron microscope were fixed with OsO_4 by addition to 0.5 ml suspension of 0.5 ml 1% OsO_4 in 0.28 M acetate-veronal buffer, pH 7.4; this mixture was allowed to stand 2 hr at 4°. After fixation the material was dialyzed overnight by the drop dialysis method [16] and then placed on a grid covered with a parlodion film. Observations were made in a UEM-100 electron microscope.

Fractionation of a homogenate from green peas. Fig. 1 shows photomicrographs made in the course of fractionation of homogenates of green seedlings. In fraction a the particles are 2.4 to 4 μ in diameter. Fraction b (3800 g, 5 min) contains particles of widely varying size. The large ones are probably chloroplasts, while the smaller may be mitochondria and grana from disintegrated chloroplasts.

Comparison of Fig. 1 with succinoxidase activity data makes evident the fact that in fraction b both plastids and mitochondria are present. Comparison of particle dimensions and succinoxidase activity distribution by fractions revealed the following:

Fractions	2300 g	3800 g	7800 g	7800 g	11 000 g	16 000 g
	5 min			15 min		
Particle dimensions, μ	2,4—4	2,4—4 0,7—1,2	0,4—1,2	0,4—1,2	Not measured.	
Succinoxidase activity, μl O_2 / mg N absorbed in 20 min	9,9	115,4	132,6	35,1	0	0

Fraction c (7800 g, 5 min) is represented by particles 1.2 μ or less in diameter, and in this fraction is concentrated the main portion of succinoxidase activity. Fraction d (7800 g, 15 min) contains particles of almost the same size, but as the figure shows, many of the particles are less dense than those of fraction c; fractions e and f are almost indistinguishable (Fig. 1). There are no structures of the mitochondrial type in these fractions. Succinoxidase activity could not be detected in fractions e and f.

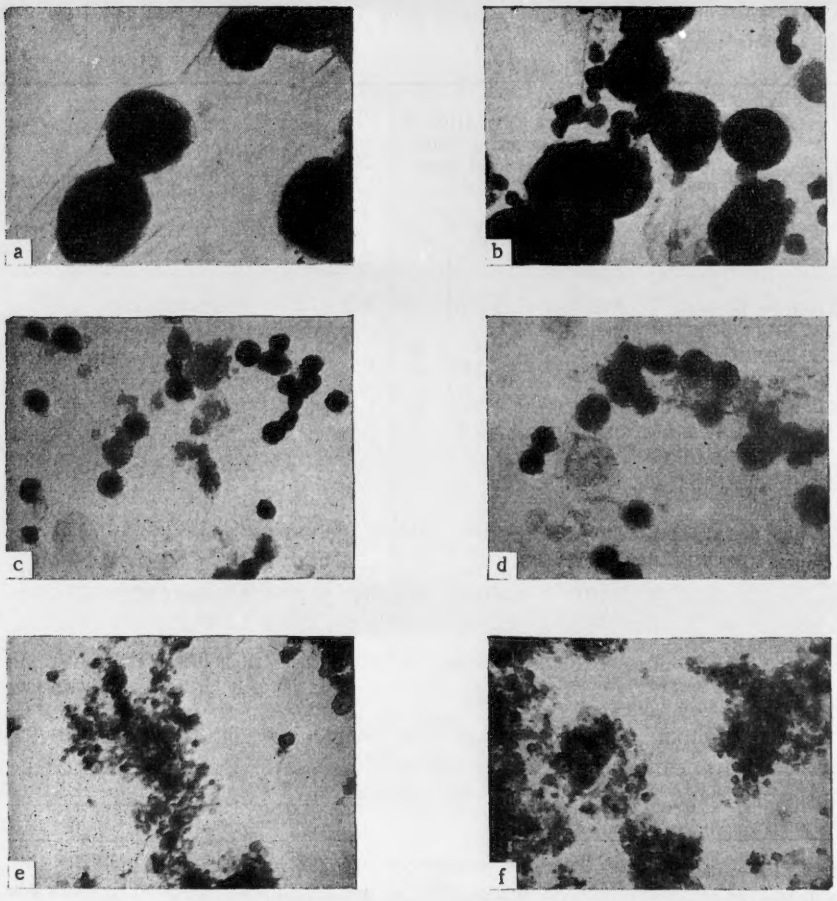

Fig. 1. Fractionation of a homogenate of green peas. For fractionation procedure, see "Methods." Magnification 6000×.

Particles with succinoxidase activity come down in fraction b (Fig. 1), but in this fraction large numbers of plastids are present. In fractions e and f mitochondria are absent. It is therefore unnecessary to employ force higher than 8000 g to obtain a mitochondrial fraction. By the method described above, however, it was not possible to obtain preparations free of contaminating chloroplast membranes. Preparations obtained are only mitochondria-enriched preparations.

Fractionation of a homogenate from etiolated peas. Fractionation of homogenates from etiolated pea seedlings by the procedure described yielded 5 fractions, the fifth fraction being obtained by centrifugation at 16,000 g for 15 min. Electron photomicrographs of these fractions are shown in Fig. 2. Comparison of particle dimensions and succinoxidase activity for the various fractions revealed the following:

Fractions	2300 *g*	3800 *g*	7800 *g*	7800 *g*	16 000 *g*
	5 min			15 min	
Particle dimensions, μ	1,4—2,7	1,4—2,4 0,5—1	0,4—1	0,3—0,8	Not measured
Succinoxidase activity, μl O_2/mg N absorbed in 20 min	0	37,9	88,7	73,7	0

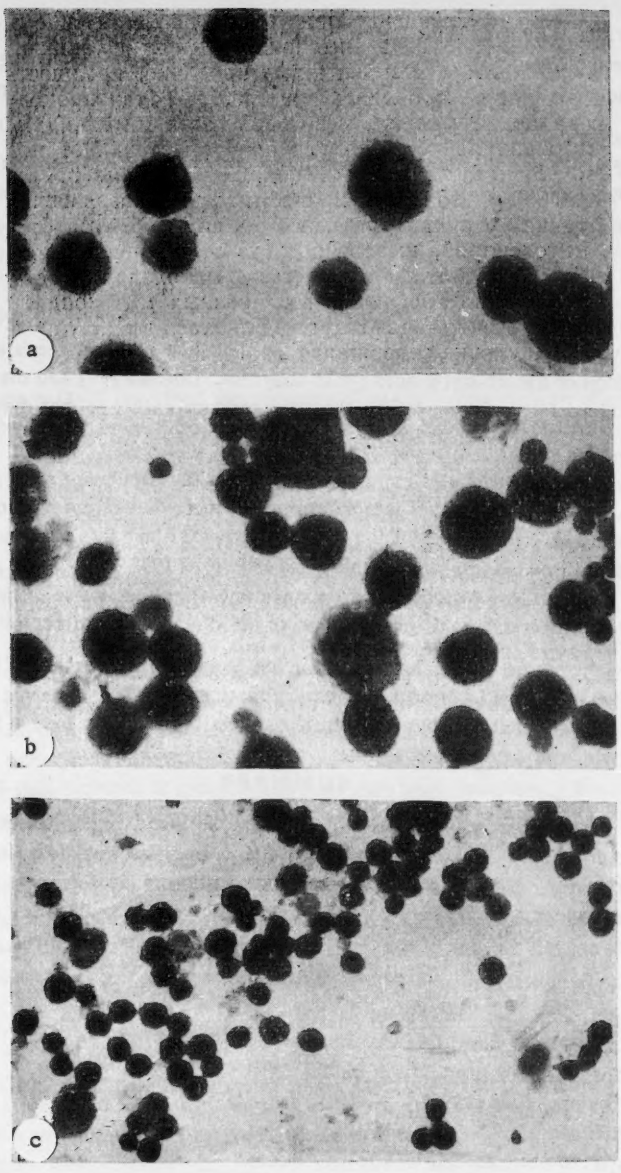

Fig. 2. Fractionation of a homogenate of etiolated peas. a— Fraction I, mainly leucoplasts; b— fraction II, leucoplasts and mitochondria; c— fraction III, mainly mitochondria. Magnification 6000×.

Fraction a is made up of leucoplasts for the most part; succinoxidase activity in this fraction is undetectable. Fraction b represents a mixture of leucoplasts and mitochondria. Succinate oxidation occurs consistently in this fraction.

The fraction obtained by centrifugation at 7800 g for 5 min contains particles from 0.4 to 1 μ in diameter. Succinoxidase activity is highest in fraction c. This fraction contains quite homogeneous particles (Fig. 2, c).

In both fraction c and fraction d leucoplasts are absent, and inasmuch as these do not have a granular structure and are less easily disrupted by homogenization than chloroplasts, grana are also absent. Thus, centrifugation at 7800 g for 5 min, or for 15 min, yields a mitochondrial fraction free of leucoplasts if these have first been

removed by centrifugation at 3800 g. This isolation procedure provides mitochondrial preparations free of leucoplast contaminants, even though some of the mitochondria are lost. It is unnecessary to use forces of 15,000 g and higher for isolation of mitochondria; indeed this can only result in contamination with structures of smaller size.

Analysis of mitochondria isolated from etiolated peas at 7800 g showed that a considerable part of this fraction is made up of lipids. Four-fold extraction of a lyophilized mitochondrial preparation with a 3 : 1 alcohol-ether mixture removed up to 40% of the dry weight. Micro-Kjeldahl determination of nitrogen in the residual material revealed that protein comprises 37% of mitochondrial dry matter.

DISCUSSION

Elucidation of the necessary conditions for isolation of plant mitochondria free of plastid admixtures affords an opportunity to resolve certain contradictions inherent in published findings. As is well known, there are various opinions as to the location of tricarboxylic acid cycle enzymes. Ohmura [17] and the authors [18] have demonstrated these enzymes in the chloroplasts. Other workers, however, adduce evidence that they are localized in mitochondrial structures [19].

As the data presented show, particles corresponding in size to mitochondria are spun down by centrifugation at 3800 g for 5 min in fairly large amounts. If it is taken into account that in our previous study chloroplasts were obtained by centrifugation at 6000 rpm, i.e., 5000 g, then it becomes clear that mitochondria must also have been present. Ohmura also employed a wide range of centrifugal forces (1000 g to 10,000 g) to obtain chloroplast fragments; in the fractions thus obtained mitochondria were present, which gave rise to the view that Krebs cycle enzymes, and also oxidative phosphorylation mechanisms, were associated with chloroplasts.

It should be noted that so far insufficient attention has been paid to removal of leucoplasts from mitochondria, where etiolated plants are being used. Preliminary centrifugation at 500 g or even at 1000 g, as is ordinarily practiced, cannot be considered adequate for obtaining mitochondria substantially free of leucoplasts.

SUMMARY

Conditions for obtaining mitochondrial preparations free of plastid contaminants by differential centrifugation of pea homogenates are described. Fractions obtained in this manner from etiolated and green pea are examined under the electron microscope. A possible explanation of contradictory findings as to location of the Krebs cycle enzymes in the cell is advanced.

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CERTAIN PROPERTIES OF PURIFIED ASPARAGINE TRANSAMINASE*

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Greenstein and co-workers [1-3] described the presence of two enzymic systems of asparagine deamination in the liver of rats. One of them is not activated by alpha-ketoacids, is thermo-labile and bound with the non-soluble particles of the liver tissue; it was named asparaginase I; the other is activated by alpha-ketoacids and was named asparaginase II [4]. Meister and co-workers [5] discovered enzymic reamination between asparagine and a number of alpha-ketoacids with the formation of oxalacetic acid and a corresponding amino acid, which is catalyzed by an enzymic liver preparation. This reaction, as demonstrated by the authors, is analogous to the reamination reaction between glutamine and alpha-ketoacids [6]. The intermediate product in the reaction of combined transamination and deamination of asparagine is the beta-amide of alpha-ketosuccinic acid [7].

In our previous works we described the obtaining of purified preparations of the transaminase of glutamine [8, 9]; certain properties of this enzyme were investigated [10]. In the course of purification of the glutamine transaminase we effected also a partial purification of asparagine transaminase by means of heating inactive proteins at 50°, fractional salting out with ammonium sulfate (0.3-0.6 saturation), electrophoresis in vertical columns filled with cellulose-powder, and subsequent adsorption on DEAE-cellulose [8, 9]. The obtained preparations of asparagine transaminase had a 40 to 46-fold increase of specific activity; they were completely freed of asparaginase, glutaminase, omega-ketoamidase, glutamine-transaminase, phenylalanine-transaminase, AL-transaminase, and partially freed of AG-transaminase.

Using purified enzymic preparations of transmainases of glutamine and asparagine, we investigated the effect of the optic isomers of cycloserine on the activity of both transaminases [11, 12], as well as the reversibility of action of both enzymes [13] and their specificity [14]. The present work is devoted to the study of certain properties of the asparagine transaminase.

EXPERIMENTAL PART

Determination of the activity of the enzyme. One-two ml test-specimens contained 1-3 mg of the asparagine transaminase protein, 7-14 micromoles of asparagine and 14-28 micromoles of pyruvate. Incubation was carried out for two hours at 37°, the proteins were then precipitated by heating in boiling water for 2-3 min and separated by centrifugation. The asparagine transaminase activity was determined according to the amount of the newly-formed (as a result of transamination) amino acid via spectrophotometric determination of its ninhydrin-copper complex after chromatography on paper [15, 16]. Neither prior, nor after a prolonged dialysis have we noted any substantial increase of the activity of the solutions of the purified enzyme upon adding pyridoxalphosphate in vitro—a fact which indicates the considerable strength of the bond between the apo- and co-enzyme.

Electrophoretic mobility of asparagine transaminase. In order to determine the electrophoretic mobility of the purified asparagine transaminase, the enzyme preparation obtained via zonal electrophoresis and subsequent adsorption of DEAE-cellulose was subjected to analysis in a Tiselius apparatus in a veronal buffer (pH 8.4, micron 0.05). Under free electrophoresis, two protein fractions were detected along the descending as well as ascending boundary. The entire asparagine transaminase activity was concentrated in the main protein peak (70% of total

* Abbreviations used in the text: DEAE-cellulose—diethylaminoethylcellulose; AL-transaminase—alanine-glutamate-transaminase; AG-transaminase—aspartate-glutamate-transaminase; ChMB—n-chloromercuribenzoate; IAA—iodoacetamide.

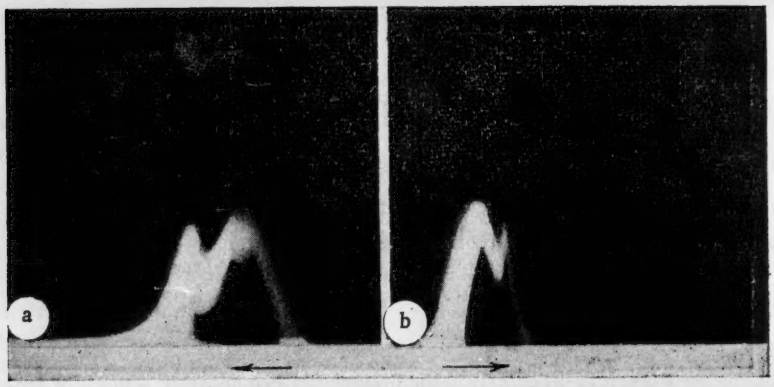

Fig. 1. An electrophoretogram of the asparagine transaminase preparation in a Tiselius apparatus. Veronal buffer, pH 8.4; micron 0.05; a—ascending boundary after 120 min; b—descending boundary after 120 min.

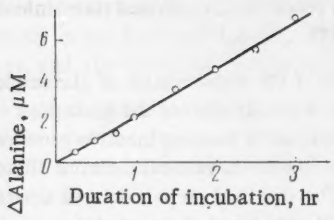

Fig. 2. Course of reaction in regard to time. The specimens contained 14 micromoles of asparagine and 28 micromoles of pyruvate in two ml 0.05 M veronal buffer, (pH 8.4); incubation at 37°.

protein), with an electrophoretic mobility equalling—2.62—2.94 · 10^{-5} · · $cm^2 \cdot sec^{-1} \cdot b^{-1}$ (along the ascending boundary in Tiselius apparatus, Fig. 1, a), or respectively + 2.61—2.95 · 10^{-5} · $cm^2 \cdot sec^{-1} \cdot b^{-1}$ (along the descending boundary, Fig. 1, b).

Course of reaction in regard to time. In the course of incubation of the enzyme with asparagine and pyruvate, the amount of forming pyruvate increases linearly for a period of three hours (Fig. 2).

The pH- optimum of asparagine transaminase. In the works of Errera and Greenstein [1, 2] it was established that the pH-optimum of deamination of asparagine in the presence of pyruvate is found at 8.0[1] or pH 6.7 [2]. These data were obtained with non-purified enzymic preparations. We established the pH-optimum of purified asparagine transaminase in its action on various substrates (pyruvate, phenylpyruvate and alpha-ketoisocapronate) by using citrate (0.05 M, pH from 4.3 to 5.5), phosphate (0.05 M, pH from 5.9 to 7.4), veronal (0.05 M, pH from 7.2 to 9.2), borate (0.025 M, pH from 9.3 to 9.9) and carbonate (0.05 M, pH from 9.2 to 10.7) buffer solutions. The results of these tests are graphically shown in Fig. 3..

As seen in Fig. 3, the pH-optimum of asparagine transaminase action with pyruvate and phenylpyruvate as an acceptor of NH_2-groups is found at 8.3; with alpha-ketoisocapronate—at 8.1. No substantial difference in the enzyme activity was found in tests with qualitatively differing buffer solutions (in the zones with overlapping pH magnitudes).

Thermal inactivation of asparagine transaminase. It is known that non-purified preparations of asparagine transaminase are comparatively thermo-stable, similar to the glutamine transaminase. We set ourselves the task of verifying the stability of purified asparagine transaminase to thermal effects. With this in view, we heated a solution of the purified enzyme at various temperatures for five minutes. The enzyme solution was then cooled to room temperature, the substrates were added and the mixtures incubated at 37°. It was found that following the heating of the enzyme at 50° the activity of the asparagine transaminase was only slightly reduced, and after heating at 80° it decreases by 63%. The effect of these and intermediate temperatures is shown in Fig. 4.

Determination of the dissociation constant of the intermediary complex of asparagine with pyruvate. In order to determine the apparent dissociation constant (K_S) of the intermediary complex of asparagine with asparagine transaminase (the Michaelis constant) we carried out experiments measuring the rate of the reamination reaction in tests containing a constant pyruvate concentration (14 · 10^{-3} M) as an acceptor, and progressively increasing asparagine concentrations (from 3.5 · 10^{-3} to 5.6 · 10^{-2} M) as donor of the NH_2-group (volume of each specimen—1 ml, incubation—two hours at 37°). According to obtained Δ-alanine magnitudes we constructed a Lineweaver-Burk diagram [17] which expresses the dependence between the reciprocal rates of the enzymic reaction (1/V) and

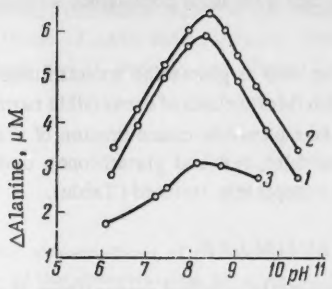

Fig. 3. pH-Optimum of action of asparagine transaminase. Test volume 2 ml, incubation 2 hr at 37°; 1) asparagine (14 micromoles) + + pyruvate (28 micromoles); 2) asparagine (14 micromoles) + phenylpyruvate (28 micromoles); 3) asparagine (14 micromoles) + alpha-ketoisocapronate (28 micromoles).

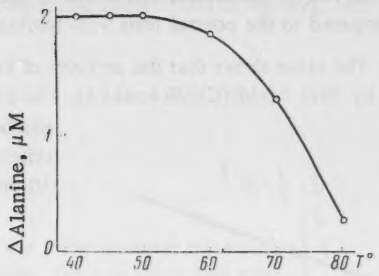

Fig. 4. Thermal inactivation of asparagine transaminase. The sample-tests contained about one mg of protein per 0.5 ml at heating time; we then added 7 micromoles of asparagine and 14 micromoles of pyruvate in 1 ml 0.05 M veronal buffer, pH 8.4, incubation two hours at 37°.

the asparagine concentration (1/S). The magnitude obtained with the use of this diagram (Fig. 5) equals $K_s = 3.33 \cdot 10^{-3}$ moles.

The effect of thiol poisons and reactivators on the asparagine transaminase activity. There are no data in the literature on the effect of thiol poisons on asparagine transaminase activity.

In our experiments, in order to elicit the effect of these inhibitors on the enzyme, the asparagine transaminase was preliminarily incubated with the inhibitors for 10 min at 25°; reactivators (thiol compounds) were then added to some of the specimens for the purpose of restoring the activity, and the preincubation was continued for 10 more minutes following which substrates were added to all specimens and incubation continued for an hour and a half at 37°.

Effect of Thiol Toxins and Reactivators on the Activity of Asparagine Transaminase. (The specimens contained 14 micromoles of asparagine and 28 micromoles of pyruvate in 2 ml of veronal buffer, pH 8.4)

Additions		Δ Alanine μM	Inhibition, %
inhibitors, M	thiol compounds (reactivators), M		
Control	—	3,62	—
—	Cysteine 10^{-4}	3,64	—
—	» $5 \cdot 10^{-4}$	3,66	—
—	Unithiol 10^{-4}	3,63	—
—	» $5 \cdot 10^{-4}$	3,67	—
—	Glutathione-SH 10^{-4}	3,62	—
—	» $5 \cdot 10^{-4}$	3,65	—
ChMB, 10^{-4}	—	1,82	49,7
» 10^{-4}	Cysteine $5 \cdot 10^{-4}$	3,35	7,5
» 10^{-4}	» 10^{-3}	3,71	—
» 10^{-4}	Unithiol 10^{-4}	3,80	—
» 10^{-4}	Glutathione-SH 10^{-4}	3,75	—
IAA, $5 \cdot 10^{-3}$	—	2,49	31,2
» $5 \cdot 10^{-3}$	Cysteine $5 \cdot 10^{-4}$	3,66	—
» $5 \cdot 10^{-3}$	» $5 \cdot 10^{-3}$	3,71	—
» $5 \cdot 10^{-3}$	Unithiol $5 \cdot 10^{-4}$	2,78	23,2
» $5 \cdot 10^{-3}$	» $5 \cdot 10^{-3}$	3,52	2,8
» $5 \cdot 10^{-3}$	Glutathione-SH $5 \cdot 10^{-4}$	3,44	5,0
» $5 \cdot 10^{-3}$	» $5 \cdot 10^{-3}$	3,57	1,4

We observed no substantial difference in the enzyme activity in the test with thiol compounds without inhibitors, as compared to the control tests with inhibitors.

The table shows that the activity of asparagine transaminase as well as glutamine transaminase [10] is inhibited by thiol toxins (ChMB and IAA). The transaminase inhibition with thiol toxins is of a reversible nature; the activity can be restored by means of an equivalent concentration of reactivators containing SH-groups (cystein, unithiol, reduced glutathione); under these conditions the activity is almost completely restored (Table).

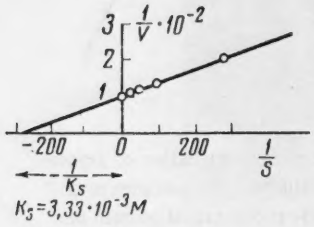

Fig. 5. Dependence of the reamination reaction rates between asparagine and pyruvate ($14 \cdot 10^{-3}$ M) on the asparagine concentration (from 3.5 to $5.6 \cdot 10^{-3}$ M). Diagram as per Lineweaver-Burk. $K_s = 3.33 \cdot 10^{-3}$ moles.

SUMMARY

In experiments with a partially purified preparations of asparagine transaminase, the electrophoretic mobility of asparagine transaminase was determined in Tiselius apparatus. It comprises in a veronal buffer, pH 8.4 (along the ascending boundary) $-2.6-2.94 \cdot 10^{-5} \cdot cm^2 \cdot sec^{-1} \cdot b^{-1}$, and (along the descending boundary) $+2.6-2.95 \cdot 10^{-5} \cdot cm^2 \cdot sec^{-1} \cdot b^{-1}$. The activity of asparagine transaminase is concentrated principally at the main protein peak which contains ~ 70% of the total protein.

The reamination reaction of asparagine with alpha-ketoacids was practically linear during the three hours. The enzyme possesses a relatively high thermo-stability: it retains ~37% of its initial activity following heating for five minutes at 80°.

The pH-optimum of purified transaminase action depends on the utilization of the aminogroup acceptor: in experiments with pyruvate it is found at pH 8.3, with phenylpyruvate at 8.4, and with alpha-ketoisocapronate at 8.1. The magnitude of the apparent dissociation of the asparagine transaminase complex with asparagine ($K_s = 3.33 \cdot 10^{-3}$ M) coincides with the dissociation magnitude of the glutamine and glutamine-transaminase complex [10]. The asparagine-transaminase activity is sharply reduced upon preincubation with thiol toxins: by 50% with 10^{-4} n-chloromercuribenzoate; by 31% with $5 \cdot 10^{-3}$ M iodoacetamide. The enzyme inhibition by these poisons can be removed with the aid of reactivators containing SH-groups (cysteine, SH-glutathione, unithiol).

Comparison of the above-cited data with the results of analogous investigations carried out with glutamine-transaminase shows that both enzymes are closely similar in their properties.

The author expresses his deep appreciation to Prof. A. E. Braunshtein for his continuous guidance in the execution of this work and he thanks V. D. Uspenskaya for her aid in carrying out the tests on free electrophoresis of the enzymic preparations.

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All abbreviations of periodicals in the above bibliography are letter-by-letter transliterations of the abbreviations as given in the original Russian journal. *Some or all of this periodical literature may well be available in English translation.* A complete list of the cover-to-cover English translations appears at the back of this issue.

WORK OF THE COMMISSION ON ENZYMES OF THE INTERNATIONAL UNION OF BIOCHEMISTRY

A. E. Braunshtein

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A vast amount of research has been devoted to the study of enzymes and the results have been published not only in the biochemical journals, but also in many chemical and biological publications. The number of known enzymes continues to grow at a rapid pace, and is not not far from a thousand. The nomenclature used by authors of research papers, monographs and textbooks for enzymes and coenzymes shows an extraordinary lack of uniformity, is in many cases obsolete, and not infrequently gives a misleading impression of the nature of the reactions catalyzed. The same terms are used, often in a single publication, to designate enzymes belonging to different classes, or on the contrary, a number of different names are used to designate one and the same enzyme. Similar confusion reigns in general enzymological terminology, and in systems of classification of enzymes, for which there exists as yet no single rational system. Neither is there any uniformity in the abbreviations and symbols used to represent the magnitudes characteristic of the kinetics of enzyme reactions, or in the principles applied to the evaluation and designation of units of enzymic activity.

This situation creates very serious difficulties in the collation and indexing of enzymological information, and, in view of the differences in the terminology current in different languages, it can often lead to misinterpretation of the meaning intended by the authors of enzymological papers.

In view of the above, the International Union of Biochemistry (IUB) established an International Commission on Enzymes in 1956, one of the most urgent tasks of which was to make recommendations for a rational system of classification of enzymes, to revise general enzymological terminology and nomenclature of individual enzymes and coenzymes, and to make recommendations concerning abbreviations and symbols used in enzyme kinetics, and for the unification of units of enzyme activity.

The following were appointed to the Commission: president—M. Dixon (U.K.), secretary—O. Hoffmann-Ostenhof (Austria); ordinary members—E. Gale (U. K.), H. Davies (U.K.), P. Desnuelle (France), S. Colowick (USA), A. Lehninger (USA), K. Linderstrom-Lang (Denmark), F. Lynen (West Germany), E. Webb (U. K.), V. Engelhardt (USSR); corresponding members—L. Leloir (Argentine), F. Egami (Japan). The following members of the Bureau of the IUB attended a number of the meetings of the Commission: M. Florkin (Blegium), R. Thompson (U.K.), E. Stotz (USA); in addition, a number of eminent enzymologists were co-opted to assist in the work of special subcommissions on the nomenclature of proteolytic enzymes, cytochromes, enzymes concerned in nitrogenous metabolism, symbols used for the description of enzyme kinetics, and units of activity.

Over 45 documents containing recommendations from members of the Commission and from other specialists, as well as a large number of orally made recommendations, were considered at five plenary sessions of the Commission (two in Paris, and one each in Vienna, Munich, and Cambridge), at meetings of the subcommissions, at joint meetings with delegates of the International Union of Pure and Applied Chemistry, at meetings with editors of biochemical journals, and by correspondence. The arduous and complex task of reaching agreement on the recommendations put before the Commission was accomplished, for the furtherance of its terms of reference [1]. The Commission submitted an interim report to the assembly of the IUB, at the IV International Biochemical Congress in Vienna (1958). At the final session of the Commission, which was held at Marseilles in 1961, its definitive recommendations were approved for publication and submission to the Assembly of the IUB at the V International Biochemical Congress (Moscow, August 1961), to the national committees of biochemists, and to the editors of biochemical journals. The recommendations are to enter into force following their approval by the Bureau of the IUB.

The Commission elaborated a unique system of classification of enzymes according to the types of reactions catalyzed, based on the decimal code system for indexing enzymes according to the types of reactions, and also agreed on a dual system of enzyme nomenclature: (a) a rational, systematic nomenclature, clearly defining the

reactions catalyzed, and (b) the ordinary, trivial name (basically, a list of enzymes as published in reference works [21]). Further, recommendations have been agreed as to general terminology and abbreviations used in enzymology, to the nomenclature of coenzymes (see the paper by M. Dixon [3], published in this issue), to standardization of units of enzyme activity, and of the symbols used in enzyme kinetics.

The arduous and responsible task of approving the recommendations of the Commission, of adapting them to the peculiar requirements of the various languages, of publishing them, and of acquainting the widest possible circles of scientists with their provisions, will rest with the national biochemical committees, the relevant scientific societies of the various countries, and the editorial boards of their biochemical journals.

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The following is a translation of an abridged Russian translation of the original paper by Dr. Dixon, the full text of which appears in Nature, Vol. 188, No. 4749, pp. 464-466, November 5, 1960.

NOMENCLATURE OF NICOTINAMIDE NUCLEOTIDE COENZYMES

M. Dixon (Cambridge)*

Translated from Biokhimiya, Vol. 26, No. 3, pp. 563-566, May-June, 1961

The conflict of views as to the nomenclature of the nicotinamide nucleotide coenzymes has long existed, and no agreement has of yet been reached. A number of systems of nomenclature of these coenzymes are, more or less widely, in current use.

Among the subjects included in the terms of reference for the consideration of the Enzyme Commission of the International Union of Biochemistry [1] was that of the nomenclature of enzymes and coenzymes. During the past three years the Commission has made a thorough and detailed search for a generally acceptable recommendation for the nomenclature of coenzymes. This question was also put before the Biological Chemistry Nomenclature Commission of the International Union of Pure and Applied Chemistry. A presentation of the nature of the problem, and of the motives which led to the adoption of the recommendations of both these Commissions, may be of interest.

Present State of the Problem

There is not at the present time any unanimity in the literature as to the nomenclature of the two coenzymes under consideration in the present paper.

Four systems of nomenclature are currently in use, to very different extents; a particular difficulty arises from the circumstance that the most widely used system is the one which gives rise to most objections on the grounds that it is misleading from the chemical point of view.

These four systems of nomenclature are as follows:

a) cozymase and phosphocozymase;
b) codehydrogenase I and codehydrogenase II (some authors from the European continent use the terms codehydrase I and codehydrase II);
c) coferment I and coferment II (or coenzyme I and coenzyme II, abbreviated to CoI and CoII);
d) diphosphopyridine nucleotide (DPN) and triphosphopyridine nucleotide (TPN).

At the present time, the first of these systems is the least, and the fourth the most widely used. None of these systems can be considered as being satisfactory: the first three because they do not convey sufficient information, and the last because it is misleading, as has been pointed out by a number of authors [2-5].

DPN and TPN. The terms diphosphopyridine nucleotide and triphosphopyridine nucleotide would be acceptable if it were not that they designate certain definite chemical structures. The basic objection to them is not only that they do not give correct information as to the structure of the coenzymes, but also that other, different compounds would be correctly described by these terms. The names in question were derived from those suggested by Warburg in 1936 [6] as convenient designations, but they are not identical with them. The term "pyridine nucleotide" was used quite legitimately for the differentiation of this class of compounds from the purine nucleotides, such as, for example, adenine nucleotides. It was only subsequently that they were named "diphospho-pyridine nucleotide" and "triphospho-pyridine nucleotide," in order to differentiate between the two coenzymes.

* The translation and the remarks are by A. E. Braunshtein, who is a member of the Commission on Enzymes; the article has been slightly shortened.

"Diphospho-." Although this term is conveniently short, it is here incorrectly used. According to the rules of chemical terminology, "phospho-X" signifies that an additional phosphate residue has been introduced into the X molecule. However, the term "nucleotide" signifies the presence in the molecule of one phosphate residue, and, accordingly, "diphospho-(di)nucleotide" should contain four phosphate residues, and not two, which are in fact present in the coenzyme molecule. Fischer has made the suggestion, based in particular on the generally accepted nomenclature of the flavin nucleotides, that it would be less ambiguous to name these coenzymes "pyridine-adenine-dinucleotide" and "Phosphopyridine-adeninedinucleotide"*; these terms would have the advantage of indicating that the compounds are dinucleotides, and that they contain adenine as a component, which the earlier terms did not indicate.

"Triphospho-." It was originally thought that the three phosphate residues of TPN were linked sequentially, as in ATP, i.e., that the two coenzymes were related to each other in the same way as ADP and ATP. When the real structure of TPN was elucidated by Kornberg and Pricer [7] in 1950, it became evident that the substance was a monophosphodinucleotide; the designation "triphospho-" must therefore be regarded as being erroneous.

"Diphosphopyridine-." Strictly speaking, this term would be applied to a nucleotide of diphosphopyridine, i.e., to a substance of the structure: diphosphopyridine–D-ribose–phosphate. It is not surprising that organic chemists often criticize biochemists for using such terms.

"Dihydrodiphospho." The undesirable prefix "dihydrodiphospho-" is attached to the terms DPN and TPN, to indicate the reduced, or dihydro form of the coenzymes; this practice is also objectionable.*

"Pyridine." The word "pyridine" should not be used to designate nucleotides, unless these are actually nucleotides of pyridine itself. The coenzymes are in fact derivatives of nicotinamide, whereas the corresponding pyridine derivatives do not function as coenzymes [8].

"DNP." This is, using the Latin alphabet, the accepted abbreviation of 2,4-dinitrophenol, a reagent which is widely used in researches dealing with the coupling of biological oxidations to phosphorylation. Papers in which the term DNP is used often refer to DPN; the similarity of these abbreviations hinders the reading of such papers, and may cause misunderstandings.

"FMN, FAD, NMH–?" The term "diphosphopyridine nucleotide" does not reveal the close analogy between this substance and the flavin nucleotides, shown in the schedule.

Schedule of Structures of the Nicotinamide and the Flavin Nucleotides

Nicotinamide mononucleotide (NMN)	Nicotinamide–D-ribose–Phosphate
Cozymase Coenzyme I (coferment I, CoI) Diphosphopyridinenucleotide (DPN) Nicotinamide-adenine-dinucleotide (NAD)	adenine–D-ribose–phosphate | nicotinamide–D-ribose–phosphate
Flavin-mononucleotide (FMN)	isoalloxazine–ribitol–phosphate
Flavin-adenine-dinucleotide (FAD)	flavin adenine–D-ribose–phosphate | isoalloxazine–ribitol–phosphate phosphate
Phospho-cozymase Coenzyme II (coferment II, CoII) Triphosphopyridinenucleotide (TPN) Nicotanamide-adenine-dinucleotide–phosphate (NADP) **	adenine–D-ribose–phosphate | nicotinamide–D-ribose–phosphate

* Apart from this, a serious inconvenience encountered in using the Cyrillic script is that in the abbreviated forms of the reduced coenzymes: and , the Latin letter H (being the symbol for hydrogen) appears side by side with the Cyrillic H (A. B.).

** See footnote on page 502.

Flavin-adenine-dinucleotide (FAD) is an analogous compound, in which instead of nicotinamide we have the flavin structure; flavin-adenine mononucleotide (FMN) being the same molecule, but without the adenine nucleotide half, and nicotinamide-mononucleotide (NMN) would similarly be the name of the coenzyme without an adenylic acid residue. These three terms, with their respective abbreviations, have been universally accepted, and no-one has suggested that they should be modified. To complete the schedule, we need to add to it the abbreviation NAD, signifying nicotinamide-adenine-dinucleotide:

FMN	NMN
FAD	NAD

The use of the abbreviation DPN instead of NAD is in conflict with the logic of this nomenclature.

To sum up the basic objections to the term "diphosphopyridine nucleotide":

a) the coenzyme is not, as is suggested by its name, a nucleotide of diphosphopyridine;

b) the name suggests the presence in the molecule of four phosphate residues, instead of two;

c) the name gives no indication that the coenzyme is a dinucleotide, or that it contains adenine;

d) the coenzyme is a nucleotide of nicotinamide, but not of pyridine; pyridine nucleotide, to which this term properly applies, has no coenzymic activity;

e) the use of this term for nicotinamide nucleotide prevents the possibility of correctly designating the pyridine analog of the coenzyme (the dinucleotide of pyridine-ribose-5-phosphate and AMP);

f) the undesirable prefix "dihydrodiphospho-" enters into the name of the reduced form of the coenzyme;

g) the name DPN is out of conformity with the series FMN, FAD, NMN, ..., and it gives no indication of its close chemical analogy with the flavin nucleotides;

h) the corresponding term "triphosphopyridine nucleotide" is indicative of a triphosphate structure, rather than of the monophosphodinucleotide structure actually possessed by this coenzyme.

Taking into consideration these weighty chemical arguments, the Enzyme Commission was unable to recommend the further retention of the DPN–TPN nomenclature system, the more so that entirely satisfactory substitutes are available for these terms. The new terms do not present any objections from the point of view of chemical nomenclature, since there is no uncertainty about the structure of the coenzyme–it is nicotinamide-adenine-dinucleotide. The only difficulties are those associated with the bringing into general use of the correct names, since the old ones have been so firmly established. The Commission realizes that it is taking a serious step in recommending the change of name, and that their recommendation will encounter a certain amount of opposition. It is, however, appropriate that at the present time, when serious efforts are being made to introduce order into enzymological terminology, the same should be done for the nomenclature of coenzymes. Some workers believe that authors should be allowed freedom of choice of whichever system of nomenclature suits their individual preferences. The Enzyme Commission has, however, been entrusted with the task of making recommendations as to the nomenclature of coenzymes. The situation is further complicated by the inclusion in the terms of reference of the Commission of a revision of enzyme nomenclature; the names of many enzymes would include those of their coenzymes. In order to avoid the necessity of recommending a multiplicity of rational names for each such enzyme, the Commission was obliged to make a choice, and to recommend one only of the possible systems of nomenclature of coenzymes.

NAD. The Commission accepted the recommendation that the CoI and DPN terminologies should be abandoned in favor of the chemically correct name, "nicotinamide-adenine-dinucleotide," abbreviated to NAD. It will readily be seen that this name is free of any of the objections listed above; it expresses the structure of the substance, it reveals its analogy to FAD, and it permits of the rational naming of its analogs. The name of its reduced form: "dihydronicotinamide-adenine-dinucleotide" correctly indicates the part of the molecule which has undergone reduction.

NADP. TPN might be named phosphonicotinamide-adenine-dinucleotide, but this would be undesirable, since the term "phosphonicotinamide" would be as misleading as the term "diphosphopyridine"; moreover, the prefix "dihydrophospho-" would again appear for the reduced form. The name recommended is "nicotinamide-adenine-dinucleotide-phosphate," shortened to NADP, and this name is free of both the above objections; the additional letter P clearly indicates that the compound has been formed from NAD by addition of a further phosphate residue.*

* The word-formation "dinucleotide-phosphate" on which this recommendation was based is somewhat awkward in Russian; it can, however, be tolerated, the more so that the Commission on Enzymes firmly recommends the use in all languages of a uniform system of terminology and abbreviation (A. B.).

Analogs of Coenzymes

The adoption of the NAD system allows of the relatively simple and unambiguous naming of coenzyme analogs. Thus, for example, the pyridine analog should be named "pyridine-adenine-dinucleotide," which can be abbreviated to PAD. The deaminated form is at present known as deaminodiphosphopyridine nucleotide; this name does not give any precise indication of the structure of this compound, since it does not indicate which part of the molecule has undergone deamination (adenine). Quite clearly, the correct name of this compound should be "nicotinamide-hypoxanthine-dinucleotide" (NHD). The TPN analog in which the ribose residue is phosphorylated at position 3' instead of the normal position 2' is now known as "3'-triphosphopyridine nucleotide." According to the new system, it would be given the rational name of "nicotinamide-adenine-dinucleotide 3'-phosphate, possibly abbreviated to NAD-3'-P. The Commission has made no official recommendations regarding the use of abbreviations of the names of analogs; the above examples are intended to serve only for illustrating the considerable advantages of the proposed new system of nomenclature.

Possible Objections

It remains to consider whether the new names recommended for the coenzymes are in full accord with the requirements of chemical terminology.

"Nucleotide." Is it altogether legitimate to include NMN and FMN among the nucleotides? If the term "nucleotide" is by definition applicable only to those substances which can be derived from nucleic acids (i. e., to the purine and pyrimidine nucleotides), it would follow that the coenzymes cannot be regarded as nucleotides, since their basic components are different. It is, however, unlikely that anyone would today insist on such a restricted interpretation of the term "nucleotide," and it would be much more reasonable to apply the term to any substance having the chemical structure: base—pentose—phosphate. The structure of NMN quite clearly satisfies this requirement. The position is not quite so clear in the case of FMN. In the first place, the FMN molecule contains ribitol in place of ribose, so that instead of the —CHOH- group of position 1 of ribose, the flavin molecule has a $-CH_2-$ group. However, the term "nucleotide" is also applied to the deoxyribonucleotides, in which the —CHOH- group at position 2 is replaced by a $-CH_2-$ group. In practice, the designation of flavin derivatives as nucleotides has gained general acceptance, and no proposals for changing their nomenclature have been advanced.

Moreover, the term "flavin" includes not only the heterocyclic nucleus, but also the ribityl residue. In view of this, the term "flavin nucleotide" should be understood to mean a nucleotide containing flavin, but not a flavin nucleotide in the same sense as NMN is a nicotinamide nucleotide.

"Dinucleotide." Finally, can NAD and FAD be regarded as dinucleotides, in the strict sense of the word? A pedant might argue that this term signifies that two mononucleotides are linked together in a given molecule in the same way as in nucleic acids, viz., by a 3',5'-phosphodiesteratic linkage. It is, however, quite reasonable to regard "dinucleotides" as being simply compounds formed by the coupling of two mononucleotides, the more so that the difference between the two types of dinucleotides in question resides solely in the point of attachment of a simple chemical bond.

It is sometimes claimed that the term "dipeptide," which refers to compounds in which the position of the linkage is precisely defined, is analogous to the term "dinucleotide," but the analogy is not a valid one. A dipeptide is not a compound of two monopeptides; the term was derived from quite different principles. A closer analogy is that provided by the term "disaccharide," which applies to compounds formed by joining together two monosaccharides, irrespective of the position of the bond joining them.

Compounds of the type of FAD have been called dinucleotides from the moment of the elucidation of their structure, and no other names have been proposed for them. This term is far more frequently encountered in the literature in the sense we attributed to it than with reference to analogous compounds in which the linkage is in the 3',5'-positions; it would now be nonsensical to attempt to restrict the application of this term to compounds of the latter type.

SUMMARY

Such are the arguments which prompted the Enzyme Commission of the International Union of Biochemistry to recommend the adoption of the terms NAD and NADP.* The Biological Chemistry Nomenclature Commission

*See Translator's footnote on page 502.

of the International Union of Pure and Applied Chemistry has examined a number of possible variants, and has also decided to recommend that these terms should supersede the currently used ones. Preliminary experience has shown that even those specialists who were initially in favor of retaining the "DPN" nomenclature very soon found that the new names were altogether satisfactory. It is to be hoped that when these names have received the official approval of both the International Unions, the relevant journals will take a leading part in their introduction.

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8. N. O. Kaplan and M. M. Ciotti, J. Biol. Chem. 221, 823 (1956).

All abbreviations of periodicals in the above bibliography are letter-by-letter transliterations of the abbreviations as given in the original Russian journal. *Some or all of this periodical literature may well be available in English translation.* A complete list of the cover-to-cover English translations appears at the back of this issue.

FIRST INTERNATIONAL COLLOQUIUM ON THE BIOCHEMISTRY OF CARBOHYDRATES GIF-SUR-YVETTE (FRANCE)

B. N. Stepanenko, E. L. Rozenfel'd, O. A. Pavlinova, and L. I. Linevich

Translated from Biokhimiya, Vol. 26, No. 3, pp. 567-568, May-June, 1961

The First International Colloquium on the Biochemistry of Carbohydrates, organized by the Centre National de Recherches Scientifiques (CNRS), was held at Gif-sur-Yvette on July 18-21, 1960.

Eminent carbohydrate specialists from a number of countries took part in the proceedings. During the four days of the colloquium, 24 program papers were read at eight sessions, reviewing the present state of some branch of carbohydrate biochemistry, and these were followed by previously submitted comments from other participants; of particular interest were the discussions following each group of papers. The greatest number of papers were from the USA, and the smallest from France and the U. K. One program paper was from the USSR (B. N. Stepanenko), and one programmed comment (E. L. Rozenfel'd).

At the session devoted to discussion of new sugars O. Luderitz (Germany) read a paper on: "The Chemistry of 3,6-Dideoxyhexoses." Over the past ten years five of the eight theoretically possible 3,6-dideoxyhexoses have been found in Nature; four of them were components of specific polysaccharides of certain bacteria, and the fifth was first found in ascarid eggs, and later also in bacteria.

A. P. MacLennan and R. Davis (U. K.) spoke on aldoheptoses and other new sugars present in the specific polysaccharides and lipopolysaccharides of bacteria. During the past ten years about 20 new sugars have been found in bacteria.

A. Gottschalk (Australia) spoke about the sialic (acylneuraminic) acids, with particular emphasis on their chemical properties (sialic acids are very widely distributed in Nature, being components of many glycoproteins and glycolipoproteins). The structure of the terminal group of the mucoprotein isolated from the maxillary salivary gland of cattle was determined; this mucoprotein has high biological activity suppressing virus agglutination of red blood corpuscles.

J. Montreuil (France) read a paper on milk oligosaccharides. During the past few years a number of new oligosaccharides have been isolated from human milk by workers from Kuhn's laboratory and by Franch authors; these include: 1) non-nitrogenous ones, containing fucose, and 2) nitrogenous ones containing N-acetylglucosamine or lactaminic acid. Some of them have been found to have an effect on the intestinal microflora, and the wholesomeness of human milk appears to be associated with the presence of these oligosaccharides. J. Bacon (U. K.), in his paper on "Oligofructosides," commented on the wide distribution in plants of three trisaccharides derived from sucrose, in which one of the three primary hydroxyl groups was in β-fructofuranosidic linkage. J. Courtois (France) dealt with the monogalactosides of sucrose, of which six of the eight possible isomers had been discovered.

I. Mering (France) and B. Lindberg (Sweden) read papers on the structure of cellulose. They pointed out that many difficult problems of the chemistry of cellulose still remain to be solved, such as the existence of chemical linkages with lignin and hemicellulose, the presence of component sugars other than glucose, and of linkages other than 1-1,4-glucosidic ones in the cellulose molecule. Thus the presence in cellulose hydrolyzates of small amounts of mannose may be due to firmly adsorbed xylans and glucomannans on the cellulose surface, rather than to the existence of chemical linkages between glucose and non-glucose residues. R. Whistler (USA) spoke on the relationships between structure and properties of polysaccharidic adhesives, which are of considerable commercial importance. In particular, he described interesting experimental work on the artificial production of new adhesives, by the chemical treatment of cheap polysaccharides, such as, for example, the introduction into the amylose molecule of hydrophilic branches of 1-acrylamido-1-deoxy-D-glucitol and other groups. A. Roudier (France), in his paper

on hemicelluloses and on the oligosides produced by their hydrolysis, discussed the problems of the nomenclature, isolation, purification, and determination of structure of the hemicelluloses.

B. N. Stepanenko (USSR) read a paper on the galactomannans and glucomannans of seeds, bulbs, and tubers. It appears that these polysaccharides fulfill the function of starch in these plants, and are of considerable practical interest in various branches of industry. Data were presented relating to the structure of a number of galactomannans and glucomannans, including the new glucomannan eremuran, discovered in the USSR.

Two sessions were devoted to problems of reactions of antibodies with antigens (polysaccharides, such as plasma substitutes, blood group polysaccharides, and microbial polysaccharides). S. Barker (U. K.) discussed the structure of the antigenic polysaccharides of pneumococci. He was able to isolate all the structural components of the polysaccharide of type V pneumococcus, and to obtain new data relating to its structure. This polysaccharide was found to contain a hitherto unknown hexosamine, termed pneumosamine. A. Staub (France) presented the results of researches on the chemistry and immunochemistry of the polysaccharides of some Salmonellae (factors O). These are highly branched polysaccharides, whose immunological specificity depends largely on the oligosaccharides present at the ends of the chains. M. MacCarthy (USA) discussed the serological specificity of the carbohydrates of the cell wall of group A streptococci. In this case, too, specificity resided in the terminal N-acetylglucosamine and rhamnose residues. M. Heidelberger's (USA) paper dealt with the problems of the relationship between serological properties and structure of the polysaccharides of salmonellas, streptococci, and pneumococci.

W. Morgan (U. K.) discussed the nature of the mucopolysaccharides responsible for the specificity of human blood groups. All these polysaccharides contain fucose residues (12-18%), and have a high content of hexosamines (25-30%). The end-groups of polysaccharides A and B have been identified, and correlations have been established between group specificities and the composition and structure of the polysaccharides concerned. E. A. Kabat (USA) reported the results of researches into the structure of dextrans, based on interference by mono- and oligo-dextrans of known structure with the dextran–antidextran precipitation reaction. E. L. Rozenfel'd (USSR) read a paper on the application of enzymic methods to the study of the structure of various dextrans, and on the relation between the length of the end-branches of their molecules and their reaction with antidextrans. D. French (USA) and D. R. Whittaker (Canada) read papers at the session devoted to the use of enzymic methods for the elucidation of the structure of starch and cellulose.

Of considerable interest were also the sessions dealing with processes of transglycosylation, which seem likely to constitute a universal mechanism for the synthesis of oligo- and poly-saccharides. J. Edelman (U. K.) spoke on transfructosylation in plants and on the enzymic processes involved in the formation of fructosans, which are accumulated in large amounts in some plants. R. Dedonde (France) read a paper entitled: "The Levansucrase of Bacillus subtilis," in which he described the isolation of this enzyme and the mechanism of its action, and K. Wallenfels (Germany) spoke on transgalactosylations catalyzed by crystalline β-galactosidase from Escherichia coli, and by β-galactosidase from calf intestinal mucosa. This author found that di- and tri-saccharides were formed by transfer of galactose from phenyl β-D-galactosides and other substrates to various acceptors, in particular to sucrose. D. Manners (U. K.) discussed the part played by α- and β-transglucosylation reactions in the enzymic formation of glycosides and oligosaccharides. J. Strominger (USA) gave a very interesting address, entitled: "Nucleotide Derivatives as Intermediate Products of Heteropolymer Synthesis." In it, he discussed the data relating to the role of nucleotide derivatives in the synthesis of chondroitin sulfates and of bacterial cell-wall polymers. The possible participation of nucleotide derivatives in the biosynthesis of cellulose was envisaged by S. Polenoel (France), in his paper: "The Biosynthesis of Cellulose."

In conclusion, we wish to record our appreciation of the excellent organization of the Colloquium, of the great hospitality of our French hosts, and of the warm, friendly atmosphere which prevailed at the Sessions and in the corridors, and which contributed to no small extent to the success of this important and very interesting scientific enterprise.

Soviet Journals Available in Cover-to-Cover Translation

ABBREVIATION	RUSSIAN TITLE	TITLE OF TRANSLATION	PUBLISHER	TRANSLATION BEGAN Vol.	Issue	Year
AÉ	Atomnaya énergiya	Soviet Journal of Atomic Energy	Consultants Bureau	1	1	1956
Akust. zh.	Akusticheskii zhurnal	Soviet Physics – Acoustics	American Institute of Physics	1	1	1955
	Antibiotiki	Antibiotics	Consultants Bureau	4	1	1959
Astr(on). zh(urn).	Astronomicheskii zhurnal	Soviet Astronomy–AJ	American Institute of Physics	34	1	1957
Avto(mat). svarka	Avtomaticheskaya svarka	Automatic Welding	British Welding Research Association (London)		1	1959
	Avtomatika i Telemekhanika	Automation and Remote Control	Instrument Society of America	27	1	1956
	Biofizika	Biophysics	National Institutes of Health*		1	1957
	Biokhimiya	Biochemistry	Consultants Bureau	21	1	1956
Byull. éksp(erim). biol. i med.	Byulleten' éksperimental'noi biologii i meditsiny	Bulletin of Experimental Biology and Medicine	Consultants Bureau	41	1	1959
DAN (SSSR) Dokl(ady) AN SSSR	Doklady Akademii Nauk SSSR	The translation of this journal is published in sections, as follows:				
	Life Sciences	Doklady Biochemistry Section	American Institute of Biological Sciences	106	1	1956
		Doklady Biological Sciences Sections (Includes: Anatomy, biophysics, cytology, ecology, embryology, endocrinology, evolutionary morphology, genetics, histology, hydrobiology microbiology, morphology, parasitology, physiology, zoology sections)	American Institute of Biological Sciences	112	1	1957
		Doklady Botanical Sciences Sections (Includes: Botany, phytopathology, plant anatomy, plant ecology, plant embryology, plant physiology, plant morphology sections)	American Institute of Biological Sciences	112	1	1957
	Chemical Sciences	Proceedings of the Academy of Sciences of the USSR, Section: Chemical Technology	Consultants Bureau	106	1	1956
		Proceedings of the Academy of Sciences of the USSR, Section: Chemistry	Consultants Bureau	106	1	1956
		Proceedings of the Academy of Sciences of the USSR, Section: Physical Chemistry	Consultants Bureau	112	1	1957
	Earth Sciences	Doklady Earth Sciences Sections (Includes: Geochemistry, geology, geophysics, hydrogeology, mineralogy, paleontology, petrography, permafrost sections)	American Geological Institute	124	1	1959
		Proceedings of the Academy of Sciences of the USSR, Section: Geochemistry	Consultants Bureau	106-123	1-6	1957-1958
		Proceedings of the Academy of Sciences of the USSR, Sections: Geology	Consultants Bureau	106-123	1-6	1957-1958
	Mathematics	Doklady Soviet Mathematics	The American Mathematics Society	131	1	1961
	Physics	Soviet Physics–Doklady (Includes: Aerodynamics, astronomy, crystallography, cybernetics and control theory, electrical engineering, energetics, fluid mechanics, heat engineering, hydraulics, mathematical physics, mechanics, physics, technical physics, theory of elasticity sections)	American Institute of Physics	106	1	1956
		Proceedings of the Academy of Sciences of the USSR, Applied Physics Sections (does not include mathematical physics or physics sections)	Consultants Bureau	106-117	1	1956-1957
Derevoobrabat. prom-st'.	Derevoobrabatyvayushchaya promyshlennost'	Wood Processing Industry	Timber Development Association (London)		9	1959
	Élecktrosvyaz	Telecommunications	Massachusetts Institute of Technology*		1	1957
Éntom(ol). oboz(renie)	Éntomologicheskoe obozrenie	Entomological Review	American Institute of Biological Sciences	38	1	1959
Farmakol. (i) toksikol(ogiya)	Farmakologiya i toksikologiya	Pharmacology and Toxicology	Consultants Bureau	20	1	1957
FMM	Fizika metallov i metallovedenie	Physics of Metals and Metallography	Acta Metallurgica*	5	1	1957
Fiziol. zhurn. SSSR (im. Sechenova)	Fiziologicheskii zhurnal im. I. M. Sechenova	Sechenov Physiological Journal USSR	National Institutes of Health*		1	1957
Fiziol(ogiya) rast.	Fiziologiya rastenii	Plant Physiology	American Institute of Biological Sciences	4	1	1957
	Geokhimiya	Geochemistry	The Geochemical Society		1	1958
FTT	Fizika tverdogo tela	Soviet Physics–Solid State	American Institute of Physics	1	1	1959
Izmerit. tekh(nika)	Izmeritel'naya tekhnika	Measurement Techniques	Instrument Society of America		1	1959
Izv. AN SSSR, O(td). Kh(im). N(auk)	Izvestiya Akademii Nauk SSSR: Otdelenie khimicheskikh nauk	Bulletin of the Academy of Sciences of the USSR: Division of Chemical Sciences	Consultants Bureau		1	1952

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Izv. AN SSSR, O(td). T(ekhn). N(auk): Met(all). i top.	(see Met. i top.)				
Izv. AN SSSR Ser. fiz(ich).	Izvestiya Akademii Nauk SSSR: Seriya fizicheskaya	Bulletin of the Academy of Sciences of the USSR: Physical Series	Columbia Technical Translations	1	1954
Izv. AN SSSR Ser. geofiz.	Izvestiya Akademii Nauk SSSR: Seriya geofizicheskaya	Bulletin (Izvestiya) of the Academy of Sciences USSR: Geophysics Series	American Geophysical Union	1	1954
Izv. AN SSSR Ser. geol.	Izvestiya Akademii Nauk SSSR: Seriya geologicheskaya	Izvestiya of the Academy of Sciences of the USSR: Geologic Series	American Geological Institute	1	1958
Kauch. i rez.	Kauchuk i rezina	Soviet Rubber Technology	Research Association of British Rubber Manufacturers	18	1 1959
	Kinetika i kataliz	Kinetics and Catalysis	Consultants Bureau	1	3 1960
	Koks i khimiya	Coke and Chemistry USSR	Coal Tar Research Association (Leeds, England)		1 1958
Kolloidn. zh(urn).	Kolloidnyi zhurnal	Colloid Journal	Consultants Bureau	14	1 1952
	Kristallografiya	Soviet Physics – Crystallography	American Institute of Physics	2	1 1957
Metalov. i term. obrabot. metal.	Metallovedenie i termicheskaya obrabotka metallov	Metal Science and Heat Treatment of Metals	Acta Metallurgica	6	1 1958
	Metallurg	Metallurgist	Acta Metallurgica		1 1957
Met. i top.	Metallurgiya i topliva	Russian Metallurgy and Fuels	Eagle Technical Publications		1 1960
Mikrobiol.	Mikrobiologiya	Microbiology	American Institute of Biological Sciences	26	1 1957
OS	Optika i spektroskopiya	Optics and Spectroscopy	American Institute of Physics		6 1959
	Pochvovedenie	Soviet Soil Science	American Institute of Biological Sciences		1 1958
	Priborostroenie	Instrument Construction	British Scientific Instrument Research Association		1 1959
Pribory i tekhn. éks(perimenta)	Pribory i tekhnika éksperimenta	Instruments and Experimental Techniques	Instrument Society of America		1 1957
Prikl. matem. i mekh.	Prikladnaya matematika i mekhanika	Applied Mathematics and Mechanics	American Society of Mechanical Engineers		1 1958
PTÉ	(see Pribory i tekhn. éks.)				
	Problemy Severa	Problems of the North	National Research Council of Canada		
Radiotekh.	Radiotekhnika	Radio Engineering	Massachusetts Institute of Technology*	12	1 1957
Radiotekh. i élektronika	Radiotekhnika i élektronika	Radio Engineering and Electronics	Massachusetts Institute of Technology*	2	1 1957
	Stanki i instrument	Machines and Tooling	Production Engineering Research Assoc.		1 1959
	Stal'	Stal (In English)	Iron and Steel Institute		1 1959
Stek. i keram.	Steklo i keramika	Glass and Ceramics	Consultants Bureau	13	1 1956
Svaroch. proiz-vo	Svarochnoe proizvodstvo	Welding Production	British Welding Research Association		4 1959
Teor. veroyat. i prim.	Teoriya veroyatnostei i ee primenenie	Theory of Probability and Its Applications	Society for Industrial and Applied Mathematics		1 1956
Tsvet. Metally	Tsvetnye metally	Nonferrous Metals	Primary Sources		1 1960
UFN	Uspekhi fizicheskikh Nauk	Soviet Physics – Uspekhi (partial translation)	American Institute of Physics	66	1 1958
UKh	Uspekhi khimii	Russian Chemical Reviews	The Chemical Society (London)		1 1960
UMN	Uspekhi matematicheskikh nauk	Russian Mathematical Surveys	London Mathematical Society	15	1 1960
Usp. fiz. nauk	(see UFN)				
Usp. khim(ii)	(see UKh)				
Usp. matem. nauk	(see UMN)				
Usp. sovr. biol.	Uspekhi sovremennoi biologii	Russian Review of Biology	Oliver and Boyd	48	1959
Vest. mashinostroeniya	Vestnik mashinostroeniya	Russian Engineering Journal	Production Engineering Research Assoc.	4	1959
Vop. gem. i per. krovi	Voprosy gematologii i perelivaniya krovi	Problems of Hematology and Blood Transfusion	National Institutes of Health*	1	1957
Vop. onk.	Voprosy onkologii	Problems of Oncology	National Institutes of Health*	1	1957
Vop. virusol.	Voprosy virusologii	Problems of Virology	National Institutes of Health*	1	1957
Zav(odsk). lab(oratoriya)	Zavodskaya laboratoriya	Industrial Laboratory	Instrument Society of America	25	1 1959
ZhAKh Zh. anal(it). khimii	Zhurnal analiticheskoi khimii	Journal of Analytical Chemistry USSR	Consultants Bureau	7	1 1952
ZhETF Zh. éksperim. i teor. fiz.	Zhurnal éksperimental'noi i theoreticheskoi fiziki	Soviet Physics–JETP	American Institute of Physics	28	1 1955
ZhFKh Zh. fiz. khimii	Zhurnal fizicheskoi khimii	Russian Journal of Physical Chemistry	The Chemical Society (London)		7 1959
ZhMÉI Zh(urn). mikrobiol. épidemiol. i immunobiol.	Zhurnal mikrobiologii, épidemiologii i immunobiologii	Journal of Microbiology, Epidemiology and Immunobiology	National Institutes of Health*		1 1957
ZhNKh Zh(urn). neorgan(ich). khim(ii)	Zhurnal neorganicheskoi khimii	The Russian Journal of Inorganic Chemistry	The Chemical Society (London)		1 1959
ZhOKh Zh(urn). obshch(ei) khimii	Zhurnal obshchei khimii	Journal of General Chemistry USSR	Consultants Bureau	19	1 1949
ZhPKh Zh(urn). prikl. khimii	Zhurnal prikladnoi khimii	Journal of Applied Chemistry USSR	Consultants Bureau	23	1 1950
ZhSKh Zh(urn). strukt. khimii	Zhurnal strukturnoi khimii	Journal of Structural Chemistry	Consultants Bureau	1	1 1960
ZhTF Zh(urn). tekhn. fiz.	Zhurnal teknicheskoi fiziki	Soviet Physics–Technical Physics	American Institute of Physics	26	1 1956
Zh(urn). vyssh. nervn. deyat. (im. Pavlova)	Zhurnal vysshei nervnoi deyatel'nosti (im. I. P. Pavlova)	Pavlov Journal of Higher Nervous Activity	National Institutes of Health*		1 1958

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